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The neurotoxicity of hallucinogenic amphetamines in primary cultures of hippocampal neurons

João Paulo Capela [a,b,*], Silvana da Costa Araújo [a], Vera Marisa Costa [a], Karsten Ruscher [c], Eduarda Fernandes [d], Maria de Lourdes Bastos [a], Ulrich Dirnagl [e], Andreas Meisel [e], Félix Carvalho [a,**]

[a] REQUIMTE (Rede de Química e Tecnologia), Laboratório de Toxicologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[b] Faculty of Health Sciences, University Fernando Pessoa, Rua Carlos da Maia, 296, 4200-150 Porto, Portugal
[c] Department of Clinical Sciences, Laboratory for Experimental Brain Research, Wallenberg Neuroscience Center, Lund University, BMC A13, S-22184 Lund, Sweden
[d] REQUIMTE (Rede de Química e Tecnologia), Laboratório de Química Aplicada, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
[e] NeuroCuro Clinical Research Center and Department of Neurology, Charité-Universitätsmedizin Berlin, Charitéplatz 1, D-10117 Berlin, Germany

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA or "Ecstasy") and 2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) are hallucinogenic amphetamines with addictive properties. The hippocampus is involved in learning and memory and seems particularly vulnerable to amphetamine's neurotoxicity.

We evaluated the neurotoxicity of DOI and MDMA in primary neuronal cultures of hippocampus obtained from Wistar rat embryos (E-17 to E-19). Mature neurons after 10 days in culture were exposed for 24 or 48 h either to MDMA (100–800 μM) or DOI (10–100 μM). Both the lactate dehydrogenase (LDH) release and the tetrazolium-based (MTT) assays revealed a concentration- and time-dependent neuronal death and mitochondrial dysfunction after exposure to both drugs. Both drugs promoted a significant increase in caspase-8 and caspase-3 activities. At concentrations that produced similar levels of neuronal death, DOI promoted a higher increase in the activity of both caspases than MDMA. In the mitochondrial fraction of neurons exposed 24 h to DOI or MDMA, we found a significant increase in the 67 kDa band of apoptosis inducing factor (AIF) by Western blot. Moreover, 24 h exposure to DOI promoted an increase in cytochrome c in the cytoplasmatic fraction of neurons. Pre-treatment with an antibody raised against the 5-HT_{2A}-receptor (an irreversible antagonist) greatly attenuated neuronal death promoted by 48 h exposure to DOI or MDMA.

In conclusion, hallucinogenic amphetamines promoted programmed neuronal death involving both the mitochondria machinery and the extrinsic cell death key regulators. Death was dependent, at least in part, on the stimulation of the 5-HT_{2A}-receptors.

1. Introduction

According to the United Nations Office on Drugs and Crime, amphetamine psychostimulants are the second major class of illicit drugs consumed for recreational purposes in the world (UNODC, 2011). Among them, 3,4-methylenedioxymethamphetamine (MDMA; "Ecstasy", "Adam", "X", "e") assumes particular importance (Capela et al., 2009).

Concerns regarding the abuse of amphetamines have been raised and neurotoxicity has been specially studied. In human volunteers, which were former "ecstasy" users, a global reduction of serotonin (5-HT) transporter binding was found in comparison to the control group. The decrease was correlated with the cumulative lifetime intake of "ecstasy" (McCann et al., 1998). Accordingly, attention and memory impairments were observed in "ecstasy" users with moderate to high misuse (Adamaszek et al., 2010), which corroborates MDMA-induced neurotoxicity in human users (Reneman et al., 2001). Importantly, in laboratory animals, MDMA promotes neurotoxic damage to 5-HT nerve endings in the forebrain, which lasts for months in rats and years in primates (Ali et al., 1993; Capela et al., 2009; Hatzidimitriou et al., 1999; Schmidt et al., 1987).

Abbreviations: 5-HT, serotonin, 5-hydroxytyptamine; 5-HT_{2A}-receptor, serotonin 2A-receptor; MDMA, 3,4-methylenedioxymethamphetamine ("Ecstasy"); DOI, (±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride; AIF, apoptosis inducing factor; Cyt c, cytochrome c; Meth, methamphetamine.

* Corresponding author at: REQUIMTE (Rede de Química e Tecnologia), Laboratório de Toxicologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. Tel.: +351 22 0428597.
** Corresponding author.
E-mail addresses: joaocapela@ff.up.pt, joaoc@ufp.pt (J.P. Capela), felixdc@ff.up.pt (F. Carvalho).

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Reports on the neurotoxic effects of amphetamines are mainly focused on the damage of serotonergic and dopaminergic systems. Nonetheless, several studies report a broader neuronal cell death induced by amphetamines, namely MDMA and methamphetamine (Meth) (Capela et al., 2009). MDMA was reported to produce neuronal degeneration in different rat brain areas such as the cortex, hippocampus, the ventromedial/ventrolateral thalamus, and the tenia tecta (Commins et al., 1987; Meyer et al., 2004; Schmued, 2003; Warren et al., 2007). Importantly, MDMA binge administration was shown to significantly decrease the survival rate of cells incorporated in the granular layer of the dentate gyrus by 50%, and of those remaining in the subgranular layer by 30%, thereby affecting the neurogenesis process (Hernandez-Rabaza et al., 2006). Also, mice chronically exposed to MDMA revealed a suppression of cell proliferation in the dentate gyrus (Cho et al., 2007). In the rat brain, heat shock protein (Hsp) 27, a molecular chaperone, and astroglial-activation (as detected by glial fibrillary acidic protein up-regulation) were found in the hippocampus CA1 region after a single dose of MDMA, which may indicate a particular susceptibility of this region (Ádori et al., 2006). Additionally, rats exposed to MDMA or Meth presented a reduction in long-term potentiation in the hippocampus (Arias-Cavieres et al., 2010; Hori et al., 2010). These results corroborate that the neurotoxicity of amphetamines is not only limited to serotonergic and dopaminergic neurons and that neuronal death occurs in the cortex, striatum and hippocampus of amphetamine treated animals (Krasnova et al., 2005). Accordingly, MDMA, Meth and related amphetamines have been shown to induce neuronal apoptosis in cultured rat dopaminergic, cortical, and cerebellar granule neurons (Capela et al., 2006b; Jimenez et al., 2004; Kanthasamy et al., 2006; Stumm et al., 1999).

2,5-Dimethoxy-4-iodoamphetamine hydrochloride (DOI) is a potent hallucinogenic amphetamine derivative. It has been used in many research studies conducted with hallucinogens since it is a prototypical potent 5-HT_{2A}-receptor agonist (Nichols, 2004). We have previously shown that DOI induced a concentration-dependent neurotoxicity in cultured cortical neurons, which could be attenuated by the 5-HT_{2A}-receptor antagonists, ketanserin and R-96544 (Capela et al., 2006b). Also, we showed that MDMA-induced cortical neuronal death could be attenuated by competitive 5-HT_{2A}-receptor antagonists and abolished by pre-treatment with the antibody raised against that receptor (Capela et al., 2007, 2006b).

Both MDMA and DOI are hallucinogenic substances that powerfully alter perception, mood, and a multitude of cognitive processes. Today, there is a general consensus on the pharmacologic mechanism of action of hallucinogens. It is believed that they exert their effects mainly through the stimulation on the serotonin 2A-receptors (5-HT_{2A}-receptors) (Nichols, 2004).

In the present manuscript, we show that the hallucinogenic amphetamines MDMA and DOI promoted mitochondrial dysfunction and neuronal death, accompanied by activation of caspase 8 and 3. Neurotoxic events involved the activation of the mitochondrial pathway with increase in the 67 kDa band of apoptosis inducing factor (AIF) in the mitochondrial fraction, and cytochrome c (cyt c) mitochondrial release. The activation of the 5-HT_{2A}-receptors was also involved in the neuronal death promoted by the drugs.

2. Materials and methods

2.1. Materials

Materials for cell cultures were obtained from the following sources: neurobasal medium and supplement B27 from Life Technologies (NY, USA); modified Eagle's medium, phosphate buffered saline (PBS), HEPES buffer, trypsin/EDTA, penicillin/streptomycin, L-glutamine, collagen-G and poly-L-lysin from Biochrom (Berlin, Germany); 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), 3,3-diaminobenzidine (DAB) enzyme-standard for kinetic lactate dehydrogenase (LDH)-test, the peptide substrate for the caspase 3 assay acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA), the peptide for caspase 3 inhibition acetyl-DEVD-CHO, the peptide substrate for the caspase 8 assay N-Acetyl-Ile-Glu-Thr-Asp-p-nitroanilide (Ac-IETD-pNA), and the protease inhibitor cocktail from Sigma–Aldrich (St. Louis, MO, USA). The drugs MDMA, DOI ((±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride) and Ketanserin were obtained from Sigma–Aldrich. R-96544 ((2R,4R)-5-[2-[2-[2-(3-methoxyphenyl)ethyl]phenoxy]ethyl]-1-methyl-3-pyrrolidinol hydrochloride) was obtained from Tocris (Bristol, UK). All other chemicals were purchased from Sigma–Aldrich. Goat polyclonal antibody raised against the 5-HT_{2A} receptor (N SC-15073) and the rabbit anti-goat biotinylated secondary antibody were purchased from Santa Cruz (CA, USA). Streptavidin-HRP was purchased from Dako A/S (Glostrup, Denmark). For Western blotting, purified mouse anti-cytochrome *c* monoclonal antibody was purchased from BD Pharmingen (San Diego, CA, USA), purified mouse anti-AIF monoclonal antibody was purchased from Santa Cruz Biotechnology (San Diego, CA, USA) and mouse anti-alpha tubulin antibody was purchased from Sigma–Aldrich (St. Louis, MO, USA). The nitrocellulose membranes (Hybond ECL), X-ray films, ECL chemiluminescence detection reagents, the anti-mouse IgG peroxidase secondary antibodies were obtained from GE Healthcare (Buckinghamshire, UK).

2.2. Cell culture

All experiments were carried out in compliance with current European directives on animal experimentation (86/609/ECC). Primary neuronal cultures of hippocampus were prepared from embryos (E-18/E-19) of Wistar rats. Cultures were prepared according to Brewer (Brewer, 1995) with minor modifications: meninges were removed, the hippocampi were dissected, and tissue was incubated for 15 min in trypsin/EDTA (0.05/0.02%, w/v in PBS) at 37 °C; the hippocampi 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 seeded out in 48-well or 6-well plates in a density of 1.1×10^5 cells/cm^2. 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. Wells were then rinsed twice with PBS before the cells were seeded in starter medium, as described previously (Capela et al., 2006a,b). Cultures were kept at 36.5 °C and 5% CO_2, 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 10th DIV.

2.3. Experimental protocol

At the 10th DIV neurons were treated with a single application of MDMA (100–800 μM). Within the following 48 h, cells were kept at 36.5 °C without feeding. MDMA concentrations were selected according to those found in the rat brain following MDMA administration. In rats administration of single MDMA doses of 20 and 40 mg/kg (s.c.) resulted in brain concentrations of approximately 206 μM (1 h after) and 466 μM (1.5 h after) respectively (Chu et al., 1996). In accordance with the allomeric principles of scaling a dose of 20 mg/kg in a rat is equivalent to a 98 mg in a

75 kg human, thus equivalent to a single pill (Ricaurte et al., 2000). It was also found that restraint stress, single housing at 15 °C or co-administration of ethanol raised the concentration of d-MDMA in the mouse brain by four- to seven-fold (Johnson et al., 2004). In fact, a multiple dosage regimen is often used in animal studies, to match the pattern of MDMA use by abusers, suggesting that this drug may accumulate in the brain following multiple drug administration (Capela et al., 2009). It seems then reasonable to believe that the concentrations used in this study are attained in vivo (Capela et al., 2006b).

In some experiments, the competitive 5-HT_{2A}-receptor antagonist ketanserin (0.1 μM) was applied to the culture 1 h before MDMA. This concentration was selected according to the drug's affinity to the receptor and, after screening experiments, its ability to provide protection, in accordance to our previous study in cortical neurons (Capela et al., 2006b). For experiments with DOI ((±)-2,5-dimethoxy-4-iodoamphetamine hydrochloride) cultures were exposed after the 10th DIV (concentration range 10–100 μM, single application without feeding for the following 48 h). DOI concentrations were selected according to their ability to produce toxicity in primary cultured cortical neurons, in accordance to our previous study (Capela et al., 2006b).

In experiments with the caspase 3 inhibitor Ac-DEVD-CHO (100 μM), the compound was applied to the cultures 1 h before the amphetamines. Drugs were diluted in medium or purified water with the exception of Ac-DEVD-CHO, which was dissolved in a DMSO containing solution (0.01% final concentration in culture medium). Cells exposed to equivalent volumes of vehicle served as controls.

The 5-HT_{2A}-receptor antibody (2 μg/mL final concentration) was used as a "non-competitive irreversible" receptor blocker, in accordance to our previous study in cortical neurons (Capela et al., 2007). The antibody was applied to the culture 1 h before the drugs in test. Since the antibodies from Santa Cruz are delivered in a buffer containing less than 0.1% of sodium azide, we tested the sole effect of this preservative in hippocampal neurons (the final concentration in the neuronal medium of sodium azide should be no higher than 0.001%). We evaluated the effect of this concentration towards neuronal viability, and verified that after 48 h exposure to 0.001% of sodium azide, neuronal cultures presented no differences towards controls in terms of LDH release (data not shown). Meanwhile, this concentration promoted significant mitochondrial dysfunction (83 ± 2% of 4 different experiments each having 4 cultured wells) relative to the respective controls, as evaluated by the MTT assay. The antibody alone (2 μg/mL final concentration in the neuronal medium) promoted an even more significant mitochondrial dysfunction (66 ± 5% of 3 different experiments each having 6 cultured wells), although not promoting LDH release. Therefore, protection experiments with the antibody were only conducted for the LDH release assays.

2.4. Cellular viability assays

Cell cultures were assessed morphologically by phase contrast microscopy at 2 different time points (24 and 48 h), as detailed bellow. Cell damage was assessed quantitatively by the measurement of LDH release into the medium (as a measure of cell membrane integrity) using a kinetic measurement assay. Also, mitochondrial dysfunction was assessed by the measurement of MTT salt metabolization assay. Finally, morphological characteristics of cell death were visualised by the fluorescent DNA-binding dyes ethidium bromide and acridine orange.

2.4.1. MTT assay

The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, based on the reduction of the tetrazolium salt is a measure of mitochondrial dysfunction. It was performed by adding 500 μg MTT/mL to neuronal cultures, followed by incubation at 36.5 °C for 35 min. The reaction was stopped by adding 10% sodium dodecyl sulphate (SDS) in 0.01 M HCl. This was followed by over-night incubation at 36.5 °C and photometric detection of formazan at 550 nm, as described previously (Capela et al., 2006a). Data obtained are presented as percentage of control vehicle treated cultures, which were set to 100%.

2.4.2. LDH assay

Cell damage was also assessed quantitatively by the measurement of 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). After exposure to drugs, aliquots (50 μL) of the medium were collected, to which 200 μl of a previously prepared 0.15 mg/mL β-NADH solution was added, at room temperature, in a 96-well microplate. Finally 25 μL pyruvate 22.7 mM was added to start the reaction. NADH oxidation to NAD^+ was measured at 340 nm, using a colorimetric 96-well plate reader (BioTek Instruments, VT, USA). LDH values are expressed in U/L, whose calculation is based on a LDH standard solution activity of 500 U/L.

2.4.3. Ethidium bromide and acridine orange staining

The fluorescent DNA-intercalating dyes ethidium bromide (EtBr) and acridine orange (AO) are suitable to distinguish live from dead cells. Also, the AO/EtBr staining allows the microscopic morphological discrimination between necrotic and apoptotic cell death (Ruscher et al., 2002). Ethidium homodimers do not penetrate intact cellular membranes. Therefore, EtBr only intercalates with nucleic acids if the outer cellular membrane has disintegrated. In contrast, the fluorescent cationic dye AO diffuse through intact membranes of live cells. When bound to DNA, it reaches an emission maximum at 525 nm. After 48 h, primary cortical neurons were incubated with 2 μg/mL AO and 2 μg/mL EtBr for 5 min before imaging, using a fluorescence microscope with a standard fluorescein excitation filter (Leica, Heerbrueg, Switzerland).

2.5. Immunocytochemistry

For immunocytochemical analysis, cells were used 48 h after incubation with MDMA (at the 12th DIV). Cells were washed with PBS and fixed with 4% paraformaldehyde in PBS at room temperature for 15 min, then rinsed with PBS, and incubated with a goat polyclonal antibody against 5-HT_{2A} (dilution 1:100) at 4 °C over night. After rinsing with PBS, cultures were exposed to a secondary rabbit biotinylated anti-goat antibody (1:250) for 1 h, followed by Streptavidin-HRP incubation. Visualization was achieved using 3,3-diaminobenzidine/H_2O_2. Omission of the primary antibody served as negative control, in which no specific staining was found.

2.6. Protein content determination

Protein content in the cellular fractions used for the Western blot assays and in total cell lysates for caspase activity assays was determined using the Bio-Rad RC DC protein assay kit, in accordance to the manufacture instructions. Stock solutions of bovine serum albumin were used as standards.

2.7. Caspase 3 and 8 assays

Measurement of caspase 3 was based on a colorimetric assay, as previously described (Jimenez et al., 2004). The hydrolysis of the peptide substrate Ac-DEVD-pNA by caspase 3 and of the peptide

substrate Ac-IETD-pNA by caspase 8 results in the release of the p-nitroaniline (pNA) moiety, which has a high absorbance at 405 nm. For each caspase activity assay, approximately 1.1×10^6 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). 50 μL of the cell lysate were added to 200 μl of the assay buffer [100 mM NaCl, 50 mM HEPES, 10 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% CHAPS (pH 7.5)]. For caspase 3 activity, the colorimetric substrate was in a final concentration of 80 μM in the assay buffer, while for caspase 8 the peptide substrate was in a final concentration of 200 μM. After incubation of the microplate at 37 °C for 24 h, absorbance was measured at 405 nm. Results of caspase 3 and 8 activities were normalized to the protein content. Caspase activities are presented as percentage of increase to the respective control cultures.

2.8. Cellular fractionation

To isolate mitochondrial and cytoplasmatic fractions for Western blot analysis of cyt c and AIF, a sucrose buffer was used as previously described (Rego et al., 2001). After scraping and collection of 3.3×10^6 cells grown in 6-well plates, these were incubated on ice with 200 μL of the buffer [(in mM): 300 sucrose, 2 EGTA, 10 HEPES (pH 7.4) to which was added 1 mM DTT, 1 mM PMSF and 2 μL (for each 200 μL of sucrose buffer) of the protease inhibitor cocktail]. The cells were lysed (2 min in ultrasonic bath) and subjected to centrifugation at $600 \times g$ for 10 min (4 °C). The supernatant was separated and submitted to additional centrifugation at $9500 \times g$ for 10 min (4 °C). The resulting supernatant was collected as cytosolic fraction, while the pellet obtained was washed with the sucrose buffer described earlier and centrifuged at $8500 \times g$ for 10 min (4 °C). The resulting pellet was dissolved in 100 μL of the complete sucrose buffer and used as mitochondrial enriched fraction.

2.9. Western immunoblot analysis of cytochrome c and AIF in cytoplasmatic and mitochondrial extracts

The levels of AIF and cyt c were determined in the cytoplasmatic and in the enriched mitochondrial extracts isolated from hipoccampus neurons. Equal amounts of proteins (40 μg) were diluted in SDS-PAGE reducing buffer [4% (w/v) SDS, 0.125 M Tris pH 6.8, 15% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 20% (v/v) mercaptoethanol] and were subjected to a SDS-polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE). The proteins in the gel were then transferred into a nitrocellulose membrane using transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.3). After washing with TBS (20 mM Tris, 0.3 mM NaCl), the membranes were then blocked for 5 h with 5% skim milk in TBS-T (20 mM Tris, 0.3 mM NaCl, 0.5% Tween 20). Primary anti-AIF and cyt c antibodies were diluted in the blocking solution to the appropriate concentration (1:500) and incubated overnight at 4 °C. After 3 washes with TBS-T, cyt c and AIF blots were incubated with anti-mouse IgG peroxidase labeled secondary antibody (1:1000) for 2 h at room temperature and then washed 3 times with TBS-T. The bands were visualized by treating the immunoblots with ECL chemiluminescence detection reagents according to the supplier's instructions, followed by exposure to X-ray film. The films were scanned and analyzed using the NIH Image J software.

2.10. Statistical analysis

Results are presented as mean ± SEM. To avoid possible variations among neuronal cultures, depending on the quality of dissection and seeding procedures, data were obtained from three to six independent experiments. Western blot data were analyzed by one-way ANOVA to compare means from different treatment groups, followed by the Student–Newman–Keuls post-hoc test, if a significant p value had been obtained. For other data 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. For pairwise comparisons, Mann–Whitney Rank Sum test was used. Statistical significance was accepted at p values less than 0.05.

3. Results

3.1. MDMA and DOI promoted neuronal death in cultured hippocampal neurons in a time- and concentration-dependent manner

Hippocampal neurons were exposed to MDMA (100–800 μM) and DOI (10–100 μM) for 48 h. Cultured neurons were assessed morphologically by phase contrast microscopy at 2 different time points (24 h and 48 h). At 24 h, there were already signs of morphological neuronal alterations, namely neurite disintegration and shrinkage of the cytoplasm, particularly at higher concentrations, but no significant differences in terms of LDH release into the medium were observed for both drugs (data not shown). Therefore, we only present data for the MTT and LDH release assay at the 48 h time-point (Fig. 1).

After 48 h exposure to MDMA it was possible to verify that MDMA neurotoxicity occurred in a concentration-dependent manner (Fig. 1A and B). Cell death, evaluated by the LDH release assay was already significant after exposure to 100 μM of MDMA, being more pronounced for the highest concentration tested (800 μM) (Fig. 1A). The MTT assay showed statistically significant mitochondrial dysfunction for all concentrations tested, being more pronounced for higher concentrations (Fig. 1B). Neurons exposed to DOI for 48 h showed more pronounced signs of toxicity for concentrations lower than those needed for MDMA (Fig. 1C and D). Cell death, evaluated by the LDH release assay was only significant after exposure to 50 μM and 100 μM of DOI, but more pronounced for the highest concentration tested (Fig. 1C). In contrast to the former assay, the MTT assay showed statistically significant mitochondrial dysfunction for all concentrations tested of DOI. Higher levels of mitochondrial dysfunction were found for the highest concentration tested (Fig. 1D).

Overall, there was a time-dependent delayed neuronal hippocampal death induced both by MDMA and DOI. Moreover, much lower concentrations of DOI were needed to induce neuronal death than those for MDMA. Additionally, both drugs promote mitochondrial dysfunction in a concentration-dependent manner.

Phase-contrast microscopy as well as ethidium bromide/acridine orange (EtBr/AO) staining were used to describe the morphological features associated with MDMA-induced neuronal death. Phase-contrast microscopy, after 48 h incubation, revealed a delayed type of neuronal death, with intensifying features of programmed neuronal death when concentrations increase. In particular, neurons exposed to increasing MDMA concentrations showed signs of neurite disintegration, shrinkage of the cytoplasm, loss of membrane integrity, and neuritic processes. To verify our microscopy observations, EtBr/AO staining was used. Through this method, living cells appear with a regular-sized green fluorescent nucleus, as shown in control neurons in Fig. 1F. After MDMA exposure we found signs of programmed neuronal death, characterized by nuclear fragmentation. In the MDMA concentrations tested (100–400 μM) we did not find neurons with necrotic signs, namely the normally sized red nucleus (Fig. 1F).

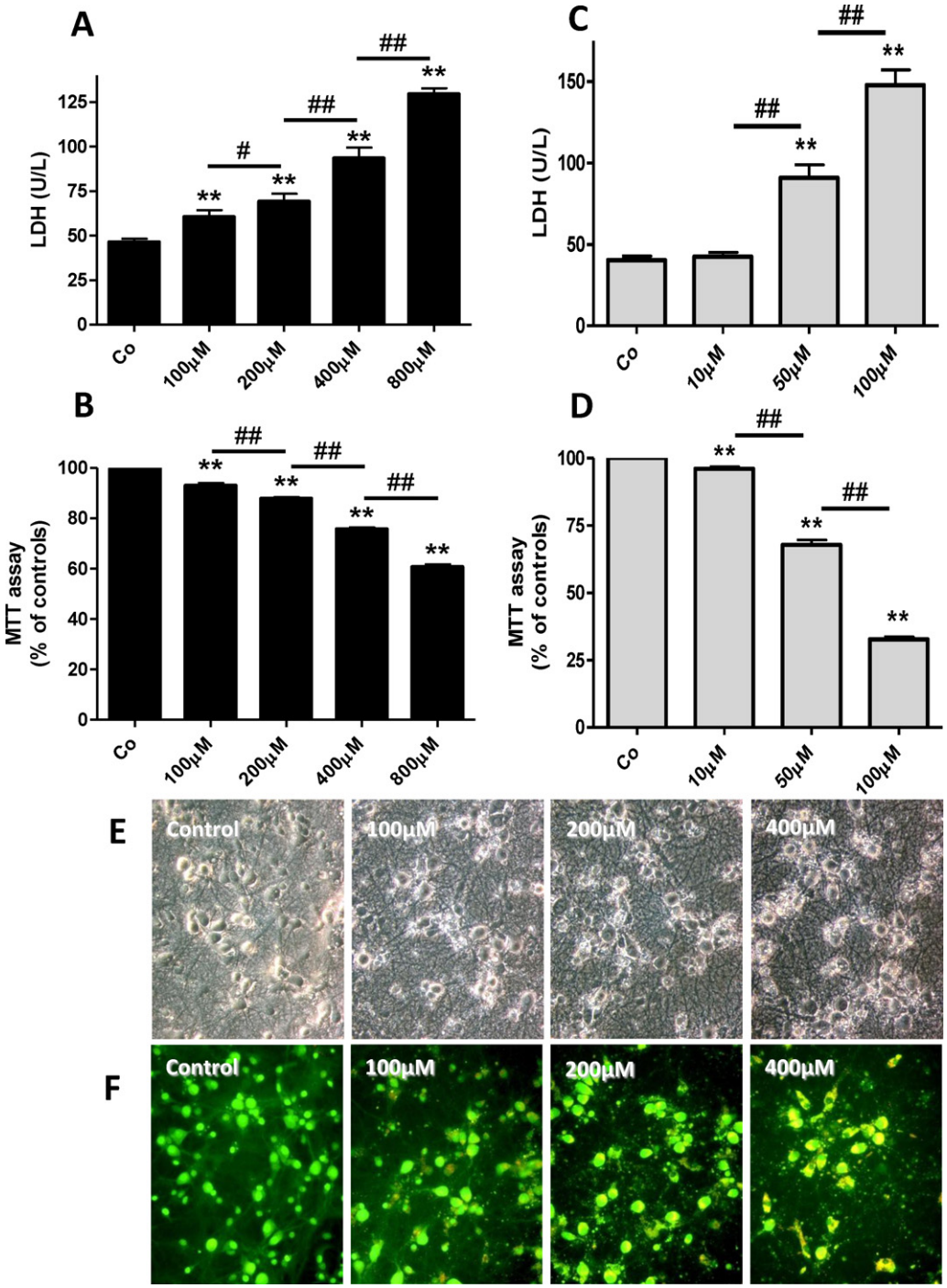

Fig. 1. MDMA and DOI-induced neurotoxicity occurred in a concentration-dependent manner. Cultures of hippocampal neurons were exposed at the 10th DIV to MDMA (100, 200, 400, and 800 μM) and DOI (10, 50, and 100 μM) and cell death was estimated after 48 h. (A and C) LDH release into the medium after MDMA and DOI exposure, respectively. (B and D) Mitochondrial dysfunction promoted by MDMA and DOI, respectively, evaluated by the MTT assay. Results were obtained from 3 different and independent experiments, each experiment having 6 different culture wells per condition (Kruskal–Wallis test followed by Student–Newman–Keuls post-hoc test, $^{**}p < 0.01$ treatment vs control, $^{\#\#}p < 0.01$ and $^{\#}p < 0.05$ treatment vs treatment). (E) Phase-contrast microscopy and (F) fluorescence microscopy of an ethidium bromide/acridine orange staining from hippocampal neurons exposed to MDMA. Note a progressive loss of neuronal branches as MDMA concentrations increase. In addition, the number of apoptotic bodies is markedly increased in MDMA stimulated cultures (magnification 400×).

3.2. MDMA- and DOI-induced neurotoxicity to hippocampal neurons was accompanied by activation of caspase 8 and 3

To assess whether neuronal death was accompanied by activation of caspase 3 and 8, we measured the activity of these caspases and verified if Ac-DEVD-CHO, a caspase 3 inhibitor attenuated neuronal death. Caspase 3 activity was significantly increased in cultured hippocampal neurons 48 h after incubation with 400 μM MDMA and 50 μM of DOI (Fig. 2A). Importantly, the increase in caspase 3 activity was significantly higher in neurons exposed to DOI when compared to MDMA.

After 48 h incubation with 400 μM MDMA, caspase 8 activity was significantly increased in cultured hippocampal neurons (Fig. 2B). Moreover, caspase 8 activity increased after

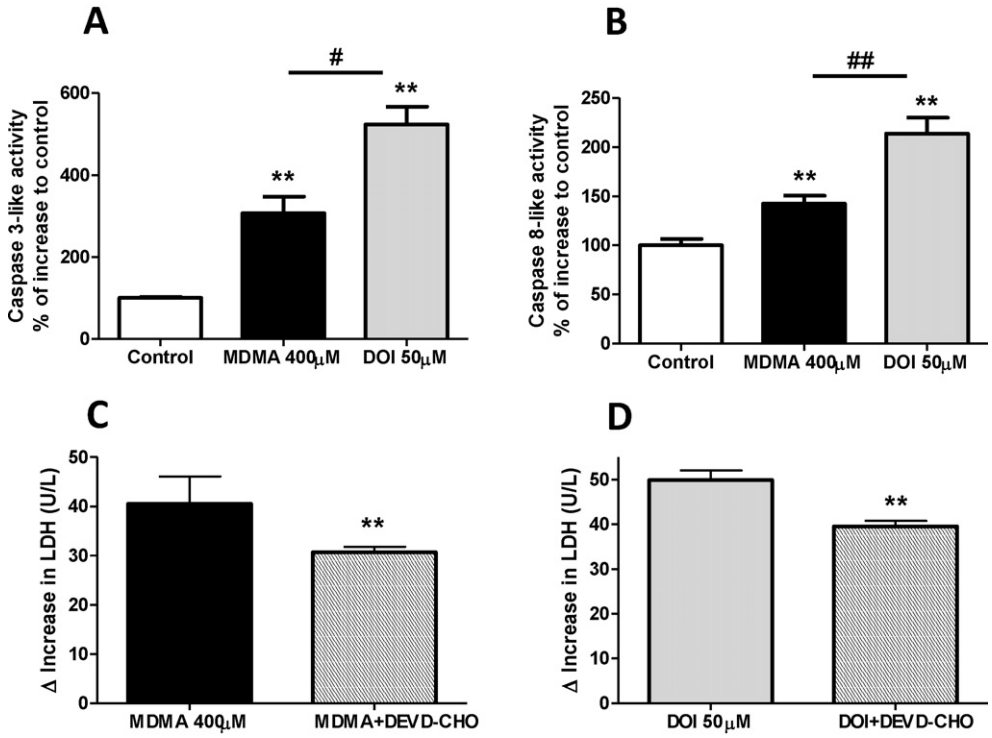

Fig. 2. MDMA and DOI-neuronal death involved caspase 8 and caspase 3 activation. (A) Caspase 3 activity in hippocampal cultures 48 h after the incubation with 400 μM of MDMA or 50 μM of DOI. Results were obtained from 3 different experiments having each experiment 2 different culture wells per condition (Kruskal–Wallis test followed by Student–Newman–Keuls post-hoc test, $^{**}p < 0.01$ treatment vs control, $^{\#}p < 0.05$ treatment vs treatment). (B) Caspase 8 activity in hippocampal cultures 48 h after the incubation with 400 μM of MDMA or 50 μM of DOI. Results were obtained from 4 different experiments having each experiment 2 different culture wells per condition (Kruskal–Wallis test followed by Student–Newman–Keuls post-hoc test, $^{**}p < 0.01$ treatment vs control, $^{\#\#}p < 0.01$ treatment vs treatment). (C and D) MDMA- and DOI-induced neurotoxicity is reduced by the presence of Ac-DEVD-CHO, a caspase-3 inhibitor, as evaluated by LDH release into the medium. Results were obtained from 3 different experiments having each experiment 6 different culture wells per condition (Mann–Whitney Rank Sum test, $^{**}p < 0.01$ treatment vs treatment).

the 48 h-incubation with 50 μM DOI. The increase of caspase 8 activity in DOI-treated neurons was significantly higher when compared to MDMA.

The caspase 3 inhibitor, Ac-DEVD-CHO (100 μM), attenuated neuronal death induced both by 400 μM of MDMA (Fig. 2C) and by 50 μM DOI (Fig. 2D), as evaluated by LDH release to the neuronal medium. No protection could be observed with the caspase 3 inhibitor against the mitochondrial dysfunction promoted by both drugs (data not shown).

3.3. AIF and cytochrome c involvement in MDMA- and DOI-induced neuronal death

We analyzed the involvement of AIF and cyt c in DOI and MDMA-induced neuronal death by analysing the cytoplasmatic and mitochondrial enriched fractions of neurons exposed to amphetamines.

The levels of mitochondrial AIF subunits 67 kDa and 57 kDa were assessed in cultured hippocampal neurons (Fig. 3A). The mitochondrial AIF subunit 67 kDa significantly increased in both DOI- and MDMA-treated neurons when compared to control (Fig. 3A), whereas the mitochondrial AIF 57 kDa bands did not significantly change when compared to the control. Moreover, there was a significant increase of the AIF subunit 67 kDa in the cytoplasmatic fraction of DOI exposed neurons. No detectable levels of the AIF 57 kDa bands could be verified in the cytoplasmatic fraction. We also performed experiments to check whether AIF was present in the nuclear fractions of exposed neurons but no detectable levels were found at the 24 h time-point. Additionally, preliminary experiments showed that there were no differences in the AIF levels of the mitochondrial, cytoplasmatic and nuclear extracts of neurons at the 48 h time-point (data not shown).

Fig. 3B shows mitochondrial and cytoplasmatic cyt c levels in all tested groups at the 24 h time-point. Only the neurons incubated with 50 μM DOI showed a significant increase in protein levels of cyt c in the cytoplasmatic fraction, when compared to the respective control. No significant changes were observed in the mitochondrial fraction in neurons treated either with DOI or MDMA, when compared to controls (Fig. 3B).

3.4. Hallucinogenic amphetamines DOI and MDMA-induced neurotoxicity involved 5-HT_{2A}-receptor activation

As illustrated in Fig. 4A hippocampal neurons in culture express the 5-HT_{2A}-receptor, as revealed by the immunocytochemistry conducted with an antibody raised against that receptor. Neurons express the 5-HT_{2A}-receptor on their cell bodies, dendrites, and axons. After exposure to 400 μM MDMA for 48 h these neurons show a dramatic reduction on the immunostaining (Fig. 4A).

To test whether MDMA and DOI-induced neurotoxicity was mediated by stimulation of the 5-HT_{2A}-receptors, we pre-incubated cultured hippocampus neurons with two types of 5-HT_{2A}-receptor antagonists. The first was an antibody directed to the 5-HT_{2A}-receptor that served as a "non-competitive irreversible antagonist", in accordance with our previous work (Capela et al., 2007). The second type was a competitive antagonist of the receptor, ketanserin.

The incubation of neurons with the 5-HT_{2A}-receptor antibody for 48 h (2 μg/mL final concentration) did not promote LDH release *per se* however it did promote mitochondrial dysfunction (as described in Section 2). As shown in Fig. 4 in panel B and C, the

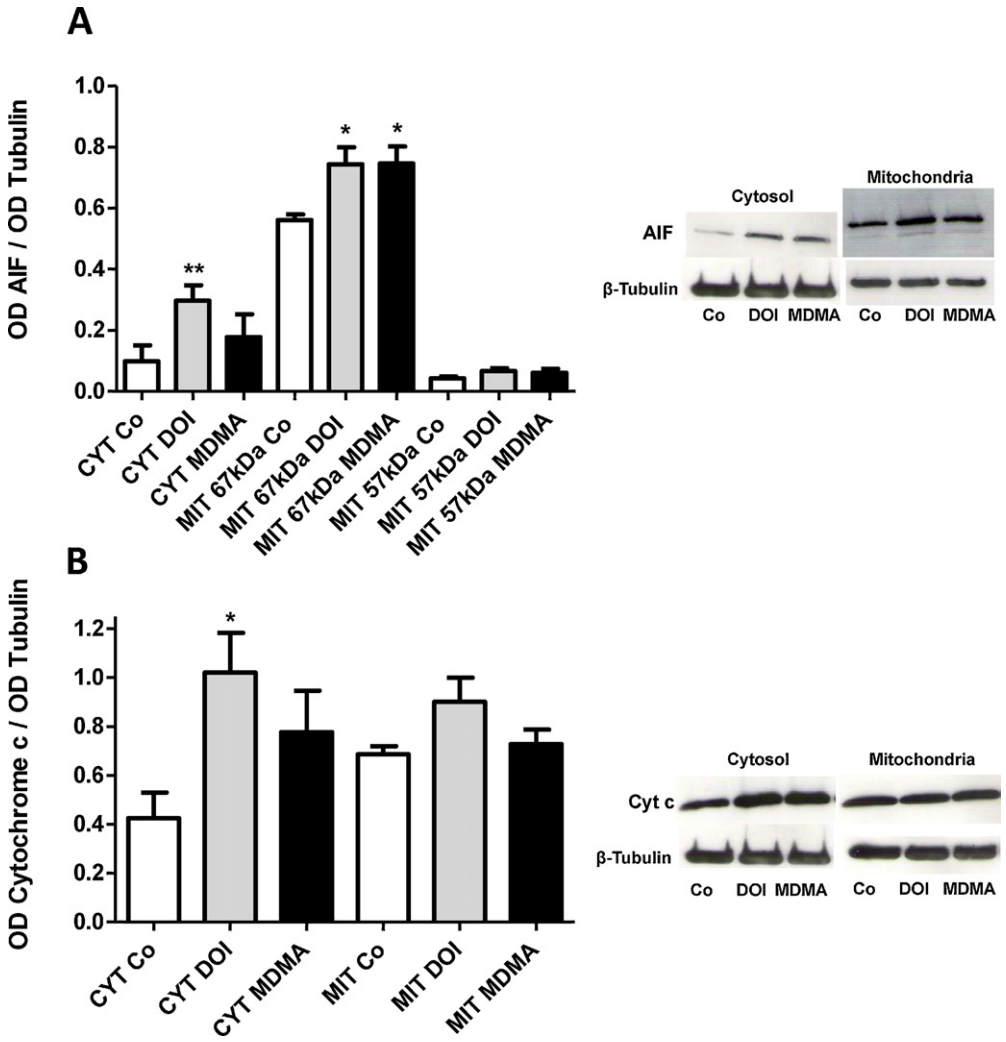

Fig. 3. (A) Western blots analysis of AIF content in the cytosolic and mitochondrial enriched fractions. (B) Western blot analysis of cytochrome c content in the cytosolic and mitochondrial fractions. The graphs represent the quantification of the blots, and in each column is presented the protein expression normalized against the loading control β-tubulin. Results were obtained from 4 to 6 independent experiments and in each experiment 3 wells per condition were collected (One-way ANOVA followed by Student–Newman–Keuls post-hoc test, $^{*}p < 0.05$ and $^{**}p < 0.01$ treatment vs control).

antibody added to the cultures 1 h prior to the exposure to 400 μM MDMA or 50 μM DOI, respectively, promoted a very significant protection against neuronal death. The antibody directed to the 5-HT_{2A}-receptor almost totally blocked the increase in LDH release. However, no protection could be verified against mitochondrial dysfunction promoted by both amphetamines (data not shown). Additionally, we observed that the morphological features of neuronal death promoted by the drugs were greatly attenuated with the antibody. As an example, we show in panel D that the features of neuronal death promoted by 50 μM of DOI, namely signs of neurite disintegration, shrinkage of the cytoplasm, loss of membrane integrity, and neuritic processes were significantly attenuated by the pre-treatment with the antibody (Fig. 4D).

To verify whether the protection obtained with the "irreversible antagonist" could also be verified with typical competitive antagonists, we performed experiments with ketanserin. In preceding experiments, the antagonist applied to cultures in concentrations up to 10 μM showed no toxic effects on cultured hippocampal neurons at 48 h (data not shown). Pre-treatment with ketanserin (0.1 μM) provided significant protection against MDMA-induced neuronal death at all tested concentrations, as shown by the LDH release assay (Fig. 4E). As with the antibody, no protection was verified against mitochondrial dysfunction (data not shown). It is important to notice that the protection against neuronal death afforded by pre-treatment with the competitive antagonist was lower when compared to the one obtained with antibody pre-treatment, the irreversible antagonist.

4. Discussion

The key findings of our study in cultured hippocampus neurons were: (1) MDMA and DOI-induced neuronal death and mitochondrial dysfunction were time- and concentration-dependent; (2) MDMA and DOI-mediated neuronal death was accompanied by activation of caspase 8 and 3; (3) neurons exposed 24 h to DOI (50 μM) or MDMA (400 μM) presented a significant increase of the 67 kDa AIF band in the mitochondrial fraction, while DOI (50 μM) also promoted an increase of cyt c in the cytoplasmatic fraction; (4) hallucinogenic amphetamines-induced cell death was dependent on 5-HT_{2A}-receptor activation, since its blockage greatly attenuated neuronal death.

To the best of our knowledge this is the first study conducted in primary hippocampal neurons to evaluate the neurotoxic features promoted by the hallucinogenic amphetamines MDMA and DOI. The present study clearly shows that both drugs promoted death and mitochondrial dysfunction to cultured hippocampal neurons.

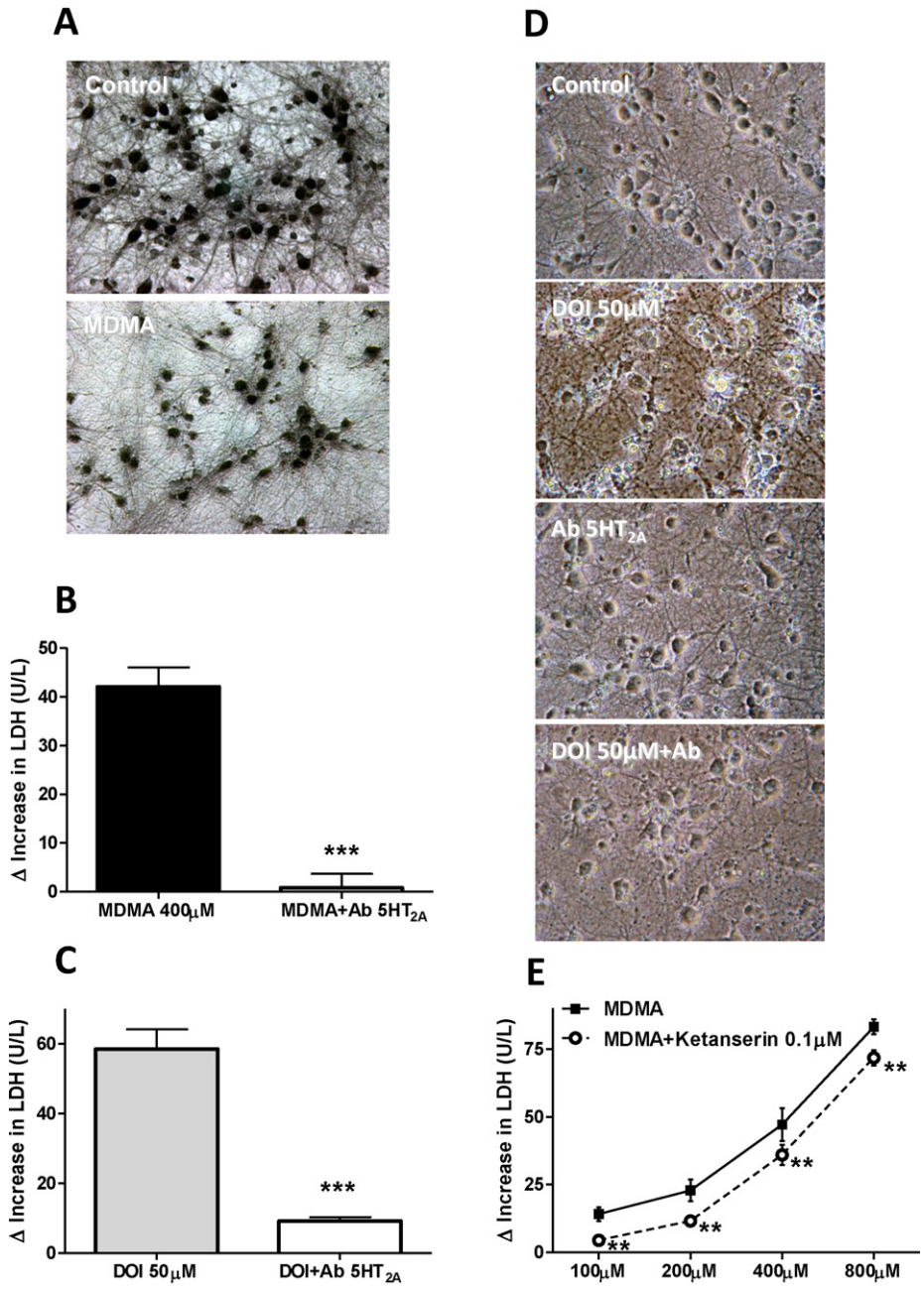

Fig. 4. MDMA and DOI-induced hippocampal neurotoxic actions were dependent on 5-HT_{2A}-receptor activation. (A) Cells containing the 5-HT_{2A}-receptor underwent neurodegeneration after MDMA exposure. There was a loss of the axons and dendrites in hippocampal neurons expressing the 5-HT_{2A}-receptor, 48 h after treatment with 400 μM MDMA, when compared to control cells (magnification 400×). (B and C) The antibody for the 5-HT_{2A}-receptor protected against MDMA and DOI-induced neurotoxicity, respectively. LDH release into the medium was evaluated, and the absolute LDH activity (U/L) values for control (vehicle treated cultures) and antibody treated cultures were: control 52.5 ± 3.2 and 5-HT_{2A}-receptor antibody 47.9 ± 3.1 (data are presented as increase of LDH release relatively to respective controls, results were obtained from 3 different experiments having each experiment 6 different culture wells per condition, Mann–Whitney Rank Sum test, $^{***}p < 0.001$ treatment vs treatment). (D) Neurons undergo neurodegeneration 48 h after 50 μM DOI exposure. The use of the 5-HT_{2A}-receptor antibody blocked this event (magnification 400×). (E) The protective effect of the competitive antagonist ketanserin (0.1 μM) against the neurotoxicity induced by MDMA (results were obtained from 3 different experiments each experiment having 6 different culture wells per condition, Kruskal–Wallis test followed by Student–Newman–Keuls post-hoc test $^{**}p < 0.01$ MDMA vs MDMA + Ketanserin).

Our findings corroborate and extend the findings of previous reports on the neurotoxicity of amphetamines to the hippocampus of animals, as highlighted in Section 1, using cultured hippocampal neurons and two hallucinogenic amphetamines describing some of the mechanisms involved.

Our findings indicate that hallucinogenic amphetamines-induced neuronal death to hippocampal neurons was accompanied both by activation of caspase 8 and caspase 3. Therefore, MDMA and DOI triggered programmed cell death mechanisms in hippocampal neurons, activating the "extrinsic" pathway. Caspase 8 is known to be recruited following activation of the receptor FADD (fas associated death domain), one of the cell death receptors. Activated caspase-8 amplifies the "death signal" by proteolytically activating downstream effector caspases, including caspase-3 (Wertz and Dixit, 2010).

We also found that DOI promoted an increase in cyt c in the cytoplasm of exposed neurons. Although there was a tendency for an increase of cytoplasmatic cyt c, no significant increase was found for MDMA exposed neurons. These results are in line with the fact that DOI was more potent in activating caspase 3 and 8

activities than MDMA. Indeed, a link has been proposed between caspase 8, which results from the "extrinsic" pathway, and cyt c mitochondrial release, which occurs in the "intrinsic" pathway of programmed cell death. This involves the caspase-8-mediated cleavage of the Bcl-2 family protein Bid to create a proapoptotic molecule, termed t-Bid (Wertz and Dixit, 2010). Ultimately t-Bid promotes mitochondrial membrane permeabilization and release of mitochondrial factors such as cyt c. Cyt c was also reported to induce proteolytically competent caspase-9 with consequent activation of downstream effector caspases, like caspase 3 (Cregan et al., 2004; Wertz and Dixit, 2010).

Mitochondria may also release factors involved in caspase-independent cell death including AIF (Krantic et al., 2007). AIF is synthesized as a 67 kDa precursor, imported into mitochondria via its N-terminal prodomain containing two mitochondrial localization sequences. Under physiological conditions, AIF remains confined to the internal mitochondrial membrane. However, upon pathological permeabilization of the outer mitochondrial membrane, mature AIF is further processed to a 57 kDa form (Krantic et al., 2007). We found an increase of the 67 kDa AIF form in the mitochondrial fraction of neurons exposed to MDMA and DOI. Also, the 67 kDa AIF form was also increased in the cytosol of DOI treated neurons. No significant changes could be observed in the 57 kDa AIF form. The increase in the mitochondria of 67 kDa AIF form can mean that AIF is being recruited from the cytosol to be processed for its involvement in cell death. Additionally, this increase can be related to the important mitochondrial functions i.e. an oxidoreductase and/or radical scavenger role has been postulated (Krantic et al., 2007). An increase of AIF in the mitochondria of hippocampal neurons is in line with the mitochondrial dysfunction promoted by both hallucinogens, therefore arguing for a possible adaptive response by recruiting AIF.

The importance of activating other pathways in programmed cell death independent of downstream effector caspases like caspase 3 for amphetamines-induced neuronal death was demonstrated by the fact that a caspase-3 inhibitor, Ac-DEVD-CHO, only partially prevented neuronal death. Given the inhibition of caspase 3 other events can be activated to conclude neuronal death. Some authors postulated that AIF can promote morphologic and biochemical events of programmed cell death thus accounting for the limited effects of caspase inhibitors (Dawson and Dawson, 2004). The mitochondrial changes of the 67 kDa AIF form observed in our work are in accordance with this assumption. Importantly, several authors showed that mitochondrial release of AIF can occur downstream of cyt c and may require caspase activation under certain conditions (Cregan et al., 2004). Therefore, AIF can serve as an additional response mechanism to facilitate the completion of cell death in certain death paradigms (Cregan et al., 2004).

In agreement with the present results in hippocampal neurons, previous reports have shown programmed neuronal death promoted by amphetamines, accompanied by activation of the mitochondrial death pathway and caspases. MDMA and Meth treatment of cultured cerebellar granule neurones produced neuronal apoptosis, accompanied by release of mitochondrial cyt c and caspase 3 activation (Jimenez et al., 2004). In primary rat cortical cultures, amphetamine decreased mitochondrial cyt c and induced activation of caspases-2, -3 and -9 with no differences in the AIF release from mitochondria (Cunha-Oliveira et al., 2006). Importantly, *in vivo* studies also reported caspase activation after exposure to amphetamines. After MDMA and Meth administration to rats, increase in caspase-3 activity was found in cortical neurons (Warren et al., 2007). MDMA treatment also produced a two-fold increase in the number of cleaved caspase-3-immunoreactive cells in the rostral forebrain and hippocampus of neonatal rats (Meyer et al., 2004). Also in mice, MDMA produced a marked induction of caspase-3 activity in the amygdala and hippocampus (Tamburini et al., 2006). Linking these results to our study, we can conclude that hallucinogenic amphetamines cause hippocampal programmed neuronal death via crosstalk between the cell death receptor pathway and the mitochondrial pathway, which caused activation of both caspase-dependent and -independent pathways.

In the rat brain hippocampus, the 5-HT_{2A}-receptor is found in many cell bodies and is responsible for many neuronal processes (Xu and Pandey, 2000). We verified that our cultivated primary hippocampal neurons also express the 5-HT_{2A}-receptor. In this study, we demonstrated that MDMA- and DOI-induced hippocampus neurotoxicity in cultured neurons was dependent on the activation of the 5-HT_{2A}-receptor. Herein, ketanserin was used in low concentrations, according to its receptor affinity, in order to preserve the specific 5-HT_{2A}-receptor antagonism (Capela et al., 2006b). Nevertheless, even with MDMA concentrations up to 8000 times higher than the competitive 5-HT_{2A}-receptor antagonists there was still a protective effect. However, pre-treatment with a 5-HT_{2A}-receptor antibody, an "irreversible" non-competitive 5-HT_{2A}-receptor blocker, almost totally blunted neuronal death promoted by DOI and MDMA. We have previously shown that MDMA and DOI-induced neurotoxicity in cortical neuronal cultures was attenuated by the selective competitive 5-HT_{2A}-receptor antagonists, ketanserin and R-96544 (Capela et al., 2006b). Also, we have reported a strong attenuation of MDMA-induced neuronal death by pre-treatment with the 5-HT_{2A}-receptor antibody in cortical neurons (Capela et al., 2007).

The affinity of MDMA and DOI towards the 5-HT_{2A}-receptor has been characterized. MDMA binding affinity for the 5-HT_{2A}-receptor presents K_i values in the range of 1–10 μM (Battaglia et al., 1988). In cells expressing the human 5-HT_{2A}- and 5HT_{2C}-receptors, the affinity of DOI for the 5-HT_{2A}-receptor was shown to lie between 0.7 and 2.4 nM (Nelson et al., 1999). These data correlate well with the fact that higher MDMA concentrations were needed to promote death of hippocampal neurons than the ones for DOI. In fact, our cell culture system lacks serotonergic input because of the absence of cells producing 5-HT and serum. Primary cultures of hippocampal neurons present mainly pyramidal neurons, dentate granule cells, and interneurons (Brewer, 1995). Therefore, it seems reasonable to conclude that DOI- and MDMA-induced neuronal death arises, at least partially, from direct stimulation of the 5-HT_{2A}-receptor.

The present findings suggest a vulnerability of the hippocampus region towards hallucinogenic amphetamines. Our results are supported both by studies conducted in animals, and also in humans. Acute MDMA treatment induces learning deficits in different animal models, namely in spatial learning and memory evaluated by the Morris water maze test, which is particularly sensitive to lesions of the hippocampus, the primary brain region believed to be involved in spatial learning and memory processing (Sprague et al., 2003). The nature of these deficits may be linked to deficits of the monoamines 5-HT and dopamine, produced by MDMA in the hippocampus (Cohen et al., 2005; Sprague et al., 2003), and also as herein shown to MDMA-induced neuronal death to hippocampal neurons. In humans, cognitive and memory deficits after frequent recreational use of "ecstasy" have been reported (McCann et al., 1999; Nulsen et al., 2011; Rodgers, 2000; van Wel et al., 2011). Several reports indicate that primary memory dysfunction in heavy "ecstasy" users may be related to a particularly high vulnerability of the hippocampus to the neurotoxic effects of MDMA (Gouzoulis-Mayfrank et al., 2003). A lower retrieval-related and more spatially restricted activity in the left anterior hippocampus was verified in MDMA abusers, and may be caused by neurotoxic actions of MDMA in this brain region (Daumann et al., 2005). Importantly, a recent study in humans demonstrated that MDMA-induced impairment of verbal memory

was mediated by 5-HT_{2A}-receptor stimulation, as pre-treatment with ketanserin could block MDMA impairment in this memory task (van Wel et al., 2011). These findings are in line with our observations that MDMA-induced neurotoxicity to hippocampal neurons was dependent on 5-HT_{2A}-receptor stimulation.

Our work reveals mechanisms by which hallucinogenic amphetamines promote hippocampal programmed neuronal death, namely through activation of caspase dependent and independent mechanisms of neuronal death, involving both the mitochondria machinery and the activation of cell death receptors. The demonstrated vulnerability of hippocampal neurons towards hallucinogens raises the concern that it may contribute to the memory dysfunction seen in heavy hallucinogenic amphetamine abusers.

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References

Adamaszek M, Khaw AV, Buck U, Andresen B, Thomasius R. Evidence of neurotoxicity of ecstasy: sustained effects on electroencephalographic activity in polydrug users. PLoS One 2010;5:e14097.

Ádori C, Andó RD, Kovács GG, Bagdy G. Damage of serotonergic axons and immunolocalization of Hsp27, Hsp72, and Hsp90 molecular chaperones after a single dose of MDMA administration in Dark Agouti rat: temporal, spatial, and cellular patterns. J Comp Neurol 2006;497:251–69.

Ali SF, Newport GD, Scallet AC, Binienda Z, Ferguson SA, Bailey JR, et al. Oral administration of 3,4-methylenedioxymethamphetamine (MDMA) produces selective serotonergic depletion in the nonhuman primate. Neurotoxicol Teratol 1993;15:91–6.

Arias-Cavieres A, Rozas C, Reyes-Parada M, Barrera N, Pancetti F, Loyola S, et al. MDMA ("ecstasy") impairs learning in the Morris water maze and reduces hippocampal LTP in young rats. Neurosci Lett 2010;469:375–9.

Battaglia G, Brooks B, Kulsakdinun C, Souza ED. Pharmacologic profile of MDMA (3,4-methylenedioxymethamphetamine) at various brain recognition sites. Eur J Pharmacol 1988;149:159–63.

Brewer GJ. Serum-free B27/neurobasal medium supports differentiated growth of neurons from the striatum, substantia nigra, septum, cerebral cortex, cerebellum, and dentate gyrus. J Neurosci Res 1995;42:674–768.

Capela JP, Carmo H, Remião F, Bastos M, Meisel A, Carvalho F. Molecular and cellular mechanisms of ecstasy-induced neurotoxicity: an overview. Mol Neurobiol 2009;39:210–71.

Capela JP, Fernandes E, Remiao F, Bastos ML, Meisel A, Carvalho F. Ecstasy induces apoptosis via 5-HT2A-receptor stimulation in cortical neurons. Neurotoxicology 2007;28:868–75.

Capela JP, Meisel A, Abreu AR, Branco PS, Ferreira LM, Lobo AM, et al. Neurotoxicity of ecstasy metabolites in rat cortical neurons, and influence of hyperthermia. J Pharmacol Exp Ther 2006a;316:53–61.

Capela JP, Ruscher K, Lautenschlager M, Freyer D, Dirnagl U, Gaio AR, et al. Ecstasy-induced cell death in cortical neuronal cultures is serotonin 2A-receptor-dependent and potentiated under hyperthermia. Neuroscience 2006b;139:1069–81.

Cho K-O, Kim S-K, Rhee GS, Kwack SJ, Cho DH, Sung KW, et al. Chronic 3,4-methylenedioxymethamphetamine treatment suppresses cell proliferation in the adult mouse dentate gyrus. Eur J Pharmacol 2007;566:120–3.

Chu T, Kumagai Y, DiStefano EW, Cho AK. Disposition of methylenodioxymethamphetamine and three metabolites in the brains of different rat strains and their possible roles in acute serotonin depletion. Biochem Pharmacol 1996;51:789–96.

Cohen AM, Skelton RM, Schaefer TL, Gudelsky GA, Vorhees C, Williams MT. Learning and memory after neonatal exposure to 3,4-methylenedioxymethamphetamine (ecstasy) in rats: interaction with exposure in adulthood. Synapse 2005;57:148–59.

Commins DL, Vosmer G, Virus RM, Woolverton WL, Schuster CR, Seiden LS. Biochemical and histological evidence that methylenedioxymethylamphetamine (MDMA) is toxic to neurons in the rat brain. J Pharmacol Exp Ther 1987;241:338–45.

Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. Oncogene 2004;23:2785–96.

Cunha-Oliveira T, Rego AC, Cardoso SM, Borges F, Swerdlow RH, Macedo T, et al. Mitochondrial dysfunction and caspase activation in rat cortical neurons treated with cocaine or amphetamine. Brain Res 2006;1089:44–54.

Daumann J, Fischermann T, Heekeren K, Henke K, Thron A, Gouzoulis-Mayfrank E. Memory-related hippocampal dysfunction in poly-drug ecstasy (3,4-methylenedioxymethamphetamine) users. Psychopharmacology 2005;180:607–11.

Dawson VL, Dawson TM. Deadly conversations: nuclear-mitochondrial cross-talk. J Bioenerg Biomembr 2004;36:287–94.

Gouzoulis-Mayfrank E, Thimm B, Rezk M, Hensen G, Daumann J. Memory impairment suggests hippocampal dysfunction in abstinent ecstasy users. Prog Neuropsychopharmacol Biol Psychiatry 2003;27:819–27.

Hatzidimitriou G, McCann UD, Ricaurte GA. Altered serotonin innervation patterns in the forebrain of monkeys treated with (+/−)3,4-methylenedioxymethamphetamine seven years previously: factors influencing abnormal recovery. J Neurosci 1999;15:5096–107.

Hernandez-Rabaza V, Dominguez-Escriba L, Barcia JA, Rosel JF, Romero FJ, Garcia-Verdugo JM, et al. Binge administration of 3,4-methylenedioxymethamphetamine ("ecstasy") impairs the survival of neural precursors in adult rat dentate gyrus. Neuropharmacology 2006;51:967–73.

Hori N, Kadota M, Watanabe M, Ito Y, Akaike N, Carpenter D. Neurotoxic effects of methamphetamine on rat hippocampus pyramidal neurons. Cell Mol Neurobiol 2010;30:849–56.

Jimenez A, Jorda EG, Verdaguer E, Pubill D, Sureda FX, Canudas AM, et al. Neurotoxicity of amphetamine derivatives is mediated by caspase pathway activation in rat cerebellar granule cells. Toxicol Appl Pharmacol 2004;196:223–34.

Johnson EA, O'Callaghan JP, Miller DB. Brain concentrations of D-MDMA are increased after stress. Psychopharmacology 2004;173:278–86.

Kanthasamy A, Anantharam V, Ali SF, Kanthasamy AG. Methamphetamine induces autophagy and apoptosis in a mesencephalic dopaminergic neuronal culture model: role of cathepsin-D in methamphetamine-induced apoptotic cell death. Ann NY Acad Sci 2006;1074:234–44.

Krantic S, Mechawar N, Reix S, Quirion R. Apoptosis-inducing factor: a matter of neuron life and death. Prog Neurobiol 2007;81:179–96.

Krasnova IN, Ladenheim B, Cadet JL. Amphetamine induces apoptosis of medium spiny striatal projection neurons via the mitochondria-dependent pathway. FASEB J 2005;19:851–3.

McCann U, Mertl M, Eligulashvili V, Ricaurte G. Cognitive performance in 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) users: a controlled study. Psychopharmacology 1999;143:417–25.

McCann UD, Szabo Z, Scheffel U, Dannals RF, Ricaurte GA. Positron emission tomographic evidence of toxic effect of MDMA ("Ecstasy") on brain serotonin neurones in human beings. Lancet 1998;352:1433–7.

Meyer JS, Grande M, Johnson K, Ali SF. Neurotoxic effects of MDMA ("ecstasy") administration to neonatal rats. Int J Dev Neurosci 2004;22:261–71.

Nelson DL, Lucaites VL, Wainscott DB, Glennon RA. Comparisons of hallucinogenic phenylisopropylamine binding affinities at cloned human 5-HT2A, 5-HT2B and 5-HT2C receptors. N-S Arch Pharmacol 1999;359:1–6.

Nichols DE. Hallucinogens. Pharmacol Ther 2004;101:131–81.

Nulsen C, Fox A, Hammond G. Electrophysiological indices of altered working memory processes in long-term ecstasy users. Hum Psychopharmacol 2011;26:488–97.

Rego AC, Vesce S, Nicholls DG. The mechanism of mitochondrial membrane potential retention following release of cytochrome c in apoptotic GT1-7 neural cells. Cell Death Differ 2001;8:995–1003.

Reneman L, Booij J, de Bruin K, Reitsma JB, de Wolff FA, Gunning WB, et al. Effects of dose, sex, and long-term abstention from use on toxic effects of MDMA (ecstasy) on brain serotonin neurons. Lancet 2001;358:1864–9.

Ricaurte GA, Yuan J, McCann UD. (±)3,4-Methylenedioxymethamphetamine ("Ecstasy")-induced serotonin neurotoxicity: studies in animals. Neuropsychobiology 2000;42:5–10.

Rodgers J. Cognitive performance amongst recreational users of "ecstasy". Psychopharmacology 2000;151:19–24.

Ruscher K, Freyer D, Karsch M, Isaev N, Megow D, Sawitzki B, et al. Erythropoietin is a paracrine mediator of ischemic tolerance in the brain: evidence from an in vitro model. J Neurosci 2002;22:10291–301.

Schmidt CJ, Levin JA, Lovenberg W. In vitro and in vivo neurochemical effects of methylenedioxymethamphetamine on striatal monoaminergic systems in the rat brain. Biochem Pharmacol 1987;36:747–55.

Schmued L. Demonstration and localization of neuronal degeneration in the rat forebrain following a single exposure to MDMA. Brain Res 2003;974:127–33.

Sprague JE, Preston AS, Leifheit M, Woodside B. Hippocampal serotonergic damage induced by MDMA (ecstasy): effects on spatial learning. Physiol Behav 2003;79:281–7.

Stumm G, Schlegel J, Schafer T, Wurz C, Mennel H, Krieg J, et al. Amphetamines induce apoptosis and regulation of bcl-x splice variants in neocortical neurons. FASEB J 1999;13:1065–72.

Tamburini I, Blandini F, Gesi M, Frenzilli G, Nigro M, Giusiani M, et al. MDMA induces caspase-3 activation in the limbic system but not in striatum. Ann N Y Acad Sci 2006;1074:377–81.

UNODC. World drug report 2011. Vienna, Austria: United Nations Office on Drugs and Crime (UNODC); 2011.

van Wel JHP, Kuypers KPC, Theunissen EL, Bosker WM, Bakker K, Ramaekers JG. Blockade of 5-HT2 receptor selectively prevents MDMA-induced verbal memory impairment. Neuropsychopharmacology 2011;36:1932–9.

Warren M, Larner S, Kobeissy F, Brezing C, Jeung J, Hayes R, et al. Calpain and caspase proteolytic markers co-localize with rat cortical neurons after exposure to methamphetamine and MDMA. Acta Neuropathol 2007;114:277–86.

Wertz IE, Dixit VM. Regulation of death receptor signaling by the ubiquitin system. Cell Death Differ 2010;17:14–24.

Xu T, Pandey SC. Cellular localization of serotonin2A (5HT2A) receptors in the rat brain. Brain Res Bull 2000;51:499–505.