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The neurotoxicity of hallucinogenic amphetamines in primary cultures of hippocampal neurons

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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.

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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.

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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 allometric principles of scaling a dose of 20 mg/kg in a rat is equivalent to a 98 mg in a

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