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Neurotoxicity of amphetamine derivatives is mediated by caspase pathway activation in rat cerebellar granule cells

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Abstract

The neurotoxic action of the abuse drugs methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA) on cerebellar granule neurones (CGNs) culture was examined. Treatment for 48 h with METH or MDMA (1–5 mM) induced a higher decrease in viability than 24 h treatment. z.VAD.fmk (100 μM) but not MK-801 nor NBQX recovered control viability values. In both cases, cell death was characterised as apoptotic rather than necrotic by morphology cell observation. Apoptosis measured by flow cytometry indicated an increase in the hypodiploid population after 48 h treatment with METH and MDMA. Apoptosis was reverted by the presence of z.VAD.fmk (100 μM) but not by 10 μM MK-801 or NBQX. Similar results were obtained by analysing nuclear chromatine condensation. These results ruled out excitotoxic participation in amphetamine derivative-induced neurotoxicity in CGNs. Participation of radical oxygen species (ROS) was evaluated using α-tocopherol (1–15 μM) and cytometric studies. The co-treatment with 4 mM METH or MDMA for 48 h partially reverted neurotoxic action and apoptotic features, indicating ROS implication in CGNs death by amphetamine derivatives. Alteration of mitochondrial function induced cytochrome C (Cyt C) release after 48-h treatment with METH and MDMA (4 mM). There was also indication of caspase-3-like activation, measured by immunoanalysis and biochemically. Finally, neurodegenerative action caused by amphetamine derivatives may be prevented by using caspase inhibitors.

Keywords: Methamphetamine; MDMA; Apoptosis; Caspases; Cerebellar granule neurones; Cytochrome C

Introduction

Abuse of illicit analogues of methamphetamine (METH), that is so-called designer drugs, is a growing problem. As they induce a subjective "high", with euphoria and increased feelings of empathy and self-esteem, these substances are currently popular drugs of abuse. Among them, the use of 3,4(±)-methylenedioxymethamphetamine (MDMA), also know as ecstasy, has increased rapidly in the last decade. The long-term consequences of such abuse are, however, poorly understood. As there have been several cases where abusers of amphetamine derivatives have presented a variety of psychiatric symptoms, some concern has been expressed about the possible neurotoxic effects of these drugs, corroborated in different studies (Green et al., 1995; Jansen, 1999; MacCann and Ricaurte, 1991; Series et al., 1994; You et al., 1999). Thus, it seems clear that recreational use of these drugs entails a risk of neurotoxicity (MacCann et al., 2000a, Parrott, 2000).

It is well established that substituted amphetamines are neurotoxic to the dopaminergic systems and other brain regions of rodents (for review, see Davidson et al., 2001). In addition to monoaminergic toxicity, METH can cause neuronal death in non-dopaminergic neurons including hippocampal remnants, as well as cortical and striatal neurones (Eisch et al., 1998; Parrot et al, 2000; Pu et al., 1996; Schmued and Bowyer, 1997). MDMA produces marked damage to striatal dopaminergic nerve terminals in mice, as evidenced by decrements in dopamine nerve terminals, dopamine metabolites, and tyrosine hydroxylase (TH) protein. MDMA is also accompanied an increase in glial fibrillary acidic protein, an astrocyte protein that serves as a marker of injury-induced gliosis (Johnson et al., 2002a, 2002b). The effect of MDMA

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on the serotoninergic function has been well reported in animal studies (for review, see Green et al., 1995). For instance, findings are reported such as a long-lasting decrease in biochemical markers for serotoninergic function (i.e. serotonin and 5-hydroxyindolacetic acid), loss of activity of the synthesis enzyme triptophanhydroxylase, and persistent reduction of [^{3}H]paroxetine-labelled serotonin uptake (Simantov and Tauber, 1997). Ricaurte et al. (2002) have described the MDMA-induced dopaminergic neurotoxicity in primates. A recent study using quantitative positron emission tomographic evidence (PET) has found evidence of a decrease in brain 5-HT transporters in human MDMA users, strongly suggesting toxic effects of "ecstasy" in serotoninergic neurons (MacCann et al., 2000b; Ricaurte et al., 2002).

Nevertheless, the cellular and molecular mechanisms involved in causing these effects have not yet been fully elucidated. In this sense, Cadet and Brannock (1998) reviewed the evidence that oxidative stress is involved in METH-induced neurotoxicity. In addition, METH administration can lead to increased extracellular glutamate concentration (Nash and Yamamoto, 1992; O'Callaghan and Miller, 2000; Yamamoto and Zhu, 1998). This excitatory neurotransmitter might cause the consequent NMDA receptor activation and involve an increased production of reactive species. In fact, glutamate receptor antagonists attenuate METH toxicity, inhibiting the fall in DA content and in TH activity (Miller and O'Callahan, 1993) and inhibiting also gliosis (Sonsalla et al., 1989, 1991). In contrast, MDMA reduces the glutamate-evoked neuronal firing and increases extracellular levels of dopamine and serotonin in the nucleus accumbens in vivo (White et al., 1994).

On the other hand, Deng and Cadet (2000) have demonstrated, in vivo, that superoxide radicals are highly implicated in METH-induced toxicity in striata of copper-zinc superoxide dismutase transgenic mice. Accumulated evidence supports the view that the toxicity of METH on the

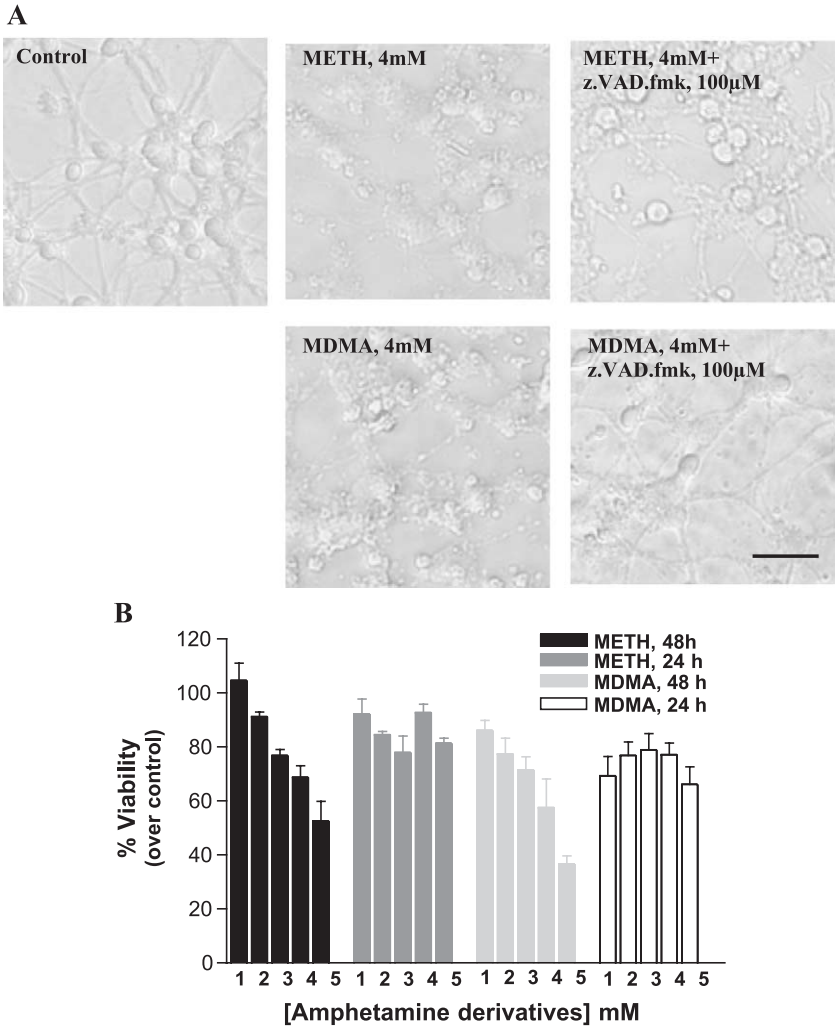

Fig. 1. Effect of METH and MDMA on the viability of CGNs for 24 or 48 h treatment. (Panel A) Representative phase contrast image of untreated cells and amphetamine derivatives treatment for 48 h in the absence or presence of z.VAD.fmk (100 µM). Calibration bar, 10 µM. (Panel B) Concentration–response curve showing the effect of exposure to amphetamine derivatives for 24 or 48 h. Data represent mean ± SEM of four to six independent experiments performed in quadruplicate.

monoaminergic system is mediated by oxygen-based radicals, irrespective of METH's mode of entry into the neurone (Davidson et al., 2001). In neuronal cells which lack monoamine transporters, METH and MDMA as a lipophilic molecule can diffuse first into the cell and then into the mitochondria and be retained there. The mitochondria have been implicated in both the necrotic and apoptotic cell death pathways. The accumulation of positively charged molecules in the cristae of the mitochondria will ultimately result in the dissipation of the electrochemical gradient established by the electron transport chain. The gradient is essential for maintaining ATP synthase and also for sustaining the integrity of the mitochondrial membrane. Both of these functions are essential for cell survival and a failure in either result in the initiation of apoptotic processes. The inhibition of the mitochondrial electron transport chain results in a massive release of calcium through the permeability transition pore and the induction of apoptosis via the cytochrome C (Cyt C) and caspase pathway. These cascades are probably directly induced by METH incorporation into mitochondria, which would disrupt the electron gradient, in a manner independent of dopamine (Davidson et al., 2001). Several authors have provided evidence that apoptosis-like events might be responsible for the deleterious effects that induces METH when administered in mammals (Cadet et al., 1997; Deng et al., 2001). Furthermore, METH modulates the expression of pro- and antiapoptotic regulators Bcl-x gene family in non-dopaminergic cell in culture (Jayanthi et al., 2001; Stumm et al., 1999).

Primary cultures of cerebellar granule neurones (CGNs) is a well-established in vitro model used to study the different pathways of death in neurones mediated by several neurotoxins as MPP^+ (Gorman et al., 1999), β-amyloid peptide (Copani et al., 1999), or glutamatergic agonists (Slagsvold et al., 2000; Verdaguer et al., 2002a, 2002b). Such primary cultures provided the most enriched in vitro system for the study of a single neuronal cell type in the central nervous system.

The main objective of this work has been to elucidate the mechanisms involved in the cell death induced by amphetamine derivatives in CGNs, a type of neurones lacking in dopamine vesicles.

Materials and methods

Cerebellar granule cell culture. Primary cultures of CGNs were prepared from pups on postnatal days 7 and 8 as previously described (Verdaguer et al., 2002a, 2002b). The cerebella from 8 to 10 rat pups were collected in 9.5 ml buffer containing in mM: 120 NaCl, 5 KCl, 25 HEPES, and 9.1 glucose. The cerebella were minced carefully with a blade and dissociated at 37 °C for 15 min with a solution containing 500 μg trypsin, 200 μg EDTA, and 500 μg DNase. The resulting solution was resuspended in basal medium Eagle's (BME) supplemented with 10% inactivated fetal calf serum (FCS), 25 mM KCl and gentamycin (50 mg/ml), and plated onto poly-L-lysine-coated 24-well plates at a density of 300 000 cells/cm^2. After 16–19 h in culture, cytosine arabinoside was added to a final concentration of 10 μM to inhibit glial cell proliferation.

Assessment of mitochondrial and cell damage. METH and MDMA toxicity in cultures of CGNs was assayed 9–10 days after plating. Cells were incubated with amphetamine derivatives at different concentrations ranging from 1 to 5 mM. The cytotoxic effects of the assayed compounds were determined 24 or 48 h later, as described below. To test the protective effect of different drugs, these compounds were added 24 h before the addition METH or MDMA. Compounds used to establish a pharmacological profile were: MK-801 and NBQX, to relieve an action through NMDA receptor and kainate/AMPA receptor, and z.VAD.fmk, to study the participation of caspase activation on METH and MDMA toxicity.

Mitochondrial damage after 24 and 48 h of METH and MDMA exposure was determined by a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tretrazolium bromide] assay according to a standard procedure (Hansen et al., 1989). The tetrazolium salt MTT is cleaved to formazan only by cells containing functional mitochondria. Thus, MTT reduction, especially in nonproliferating cells such as CGNs, is an indicator of cellular viability (Ankarcrona, 1998; Harada and Sugimoto, 1997). Results obtained from the MTT assay directly correlated with mitochondrial function and with the extent of the cell death in contrast with control conditions.

We also measured cellular and nuclear morphology after incubation of amphetamine derivatives. CGNs were grown on polylisinized cover slides and were observed with a phase contrast microscopy using the 20× lens (Nikon

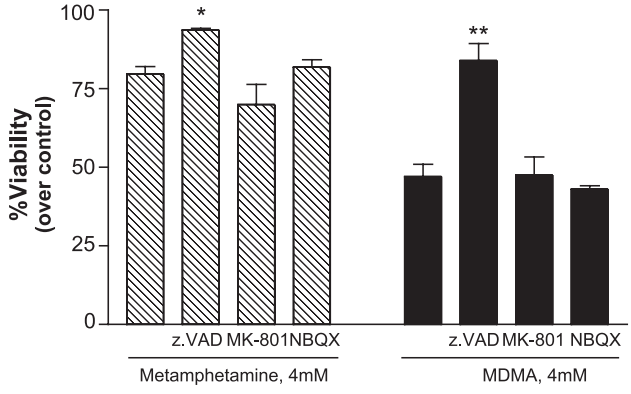

Fig. 2. Effect on cell viability of z.VAD.fmk (100 μM), MK-801 (10 μM), and NBQX (10 μM) on amphetamine derivatives-induced toxicity. CGNs were pretreated with drugs 30 min before METH (4 mM) or MDMA (4 mM) addition for 48 h. The data represent the mean ± SEM of three to four MTT independent experiments ruled out in quadruplicate and are expressed as the percentage change relative to control cells arbitrary set at 100%. The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test; $*P < 0.05$, $**P < 0.01$ vs. amphetamine derivatives.

Eclipse, TE 200, Nikon Instech Corp., Japan) after 24–48 h of amphetamine derivative treatment. Digital images were collected and filed.

Radical oxygen species (ROS) production induced by amphetamine derivatives measured by laser scanning cytometer. The formation of intracellular ROS was measured 2′,7′-dichlorofluorescein diacetate (DCFH-DA). Viable cells deacetylate DFCH-DA to 2′,7′-dichlorofluorescein, which is not fluorescent, but reacts quantitatively with ROS within cell to produce the fluorescent dye 2′,7′-dichlorofluorescein (DCF), which remains trapped within the cell and can be measured to provide an index of intracellular oxidation. Fluorescence was measured using a laser scanning cytometer (LSC, CompuCyte, Cambridge, MA) equipped with a krypton/argon laser operating at 488 and 568 nm. CGNs were grown on poly-L-lysine-coated glass coverslips and treated for 24 h with 4 mM METH or MDMA in the presence or absence of α-tocopherol (10 μM) as described above. To assess the fluorescence intensity of cells, cover slides were incubated for at least 10 min with DCFH-DA (10 μM), cells were mounted on a glass slide and subjected to LSC. A single red fluorescence detector (525 nm) was used in all the experiments and the fluorescence of 2500 CGNs in each experiment was measured. Results were generated using integral computational and display software (Compusort and Wincyte) and presented as a mean fluorescence intensity.

Analysis of apoptosis induced by amphetamine derivatives. Propidium iodide (PI) staining was used to detect morphological evidence of apoptosis as nuclear fragmentation or

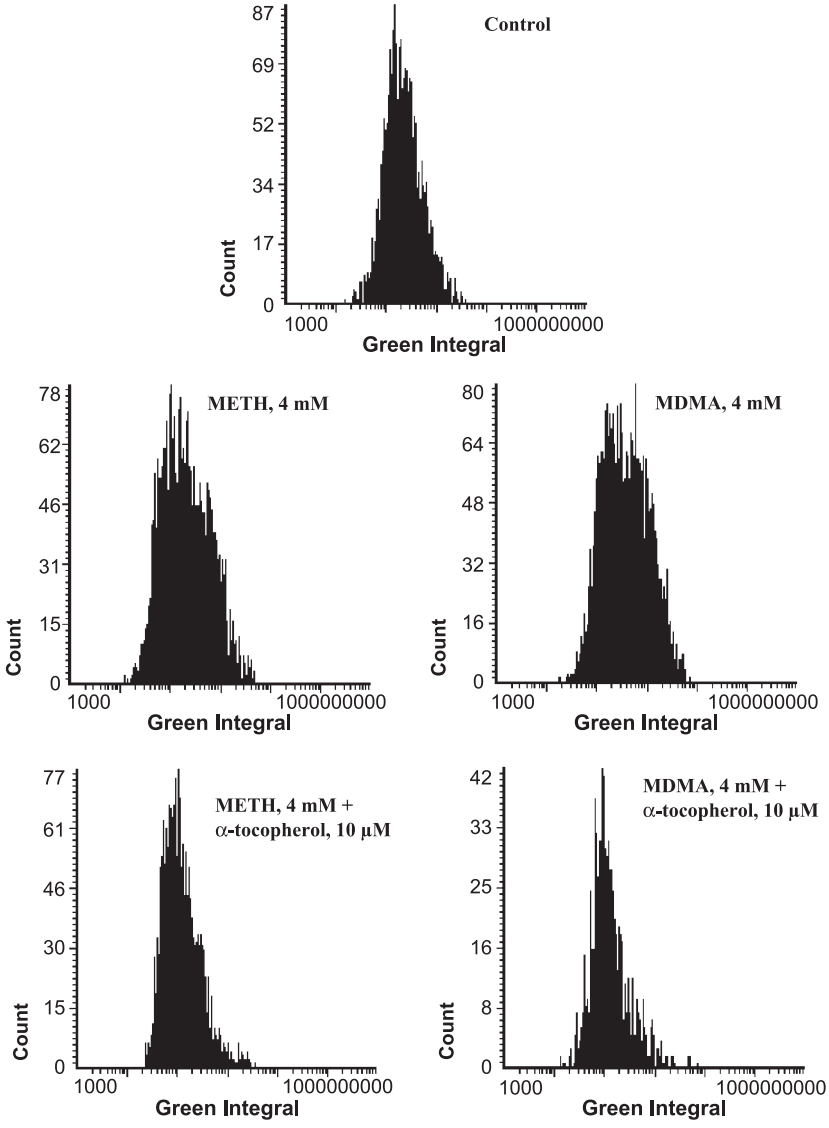

Fig. 3. Laser scanning histograms showing the ROS production induced by 4 mM amphetamine derivatives and the inhibition by α-tocopherol (10 μM). The histogram ordinate is the number of cells at each fluorescence at each 2′,7′-DCFH fluorescence intensity. Histograms are each one representative of those obtained from three different experiments carried out in triplicate.

chromatin condensation. To determine the fraction of condensed nuclei, a feature of apoptosis, CGNs were fixed in 4% paraformaldehyde in PBS for 60 min. After washing with phosphate buffer solution (PBS), they were incubated for 3 min with a solution of PI in PBS (10 μg/ml). After washing, stained cells were visualised under fluorescent light (Eclipse TE 200, Nikon Instech Corp.) and their digitised images were captured. The apoptotic cells resulted in the shrunken, brightly fluorescent, apoptotic nuclei showing high fluorescence and condensed chromatin compared with non-apoptotic cells. Apoptotic cells were scored by counting at least 500 cells in four different fields for each sample in three different experiments.

Apoptosis was also measured by flow cytometry. After 48 h METH or MDMA treatment, the culture medium was removed, cells were washed with PBS and collected from culture plates by gently pipetting. PI (10 μg/ml) was added 30 min before cytofluorometry analysis. Flow cytometer experiments were carried out using Epics XL flow cytometer (Beckman Coulter Corp., Florida, USA). The instrument was set up with the standard configuration: excitation of the sample was done using as a standard 488-nm air-cooled argon-ion laser at 15 mW power. Forward scatter (FSC), side scatter (SSC), and red (620 nm) fluorescence for PI were acquired. Optical alignment was based on the optimised signal from 10-nm fluorescent beads. Red fluorescence was projected on a 1024 monoparametrical histogram. Aggregates were excluded by gating single cells by their area vs. peak fluorescence signal.

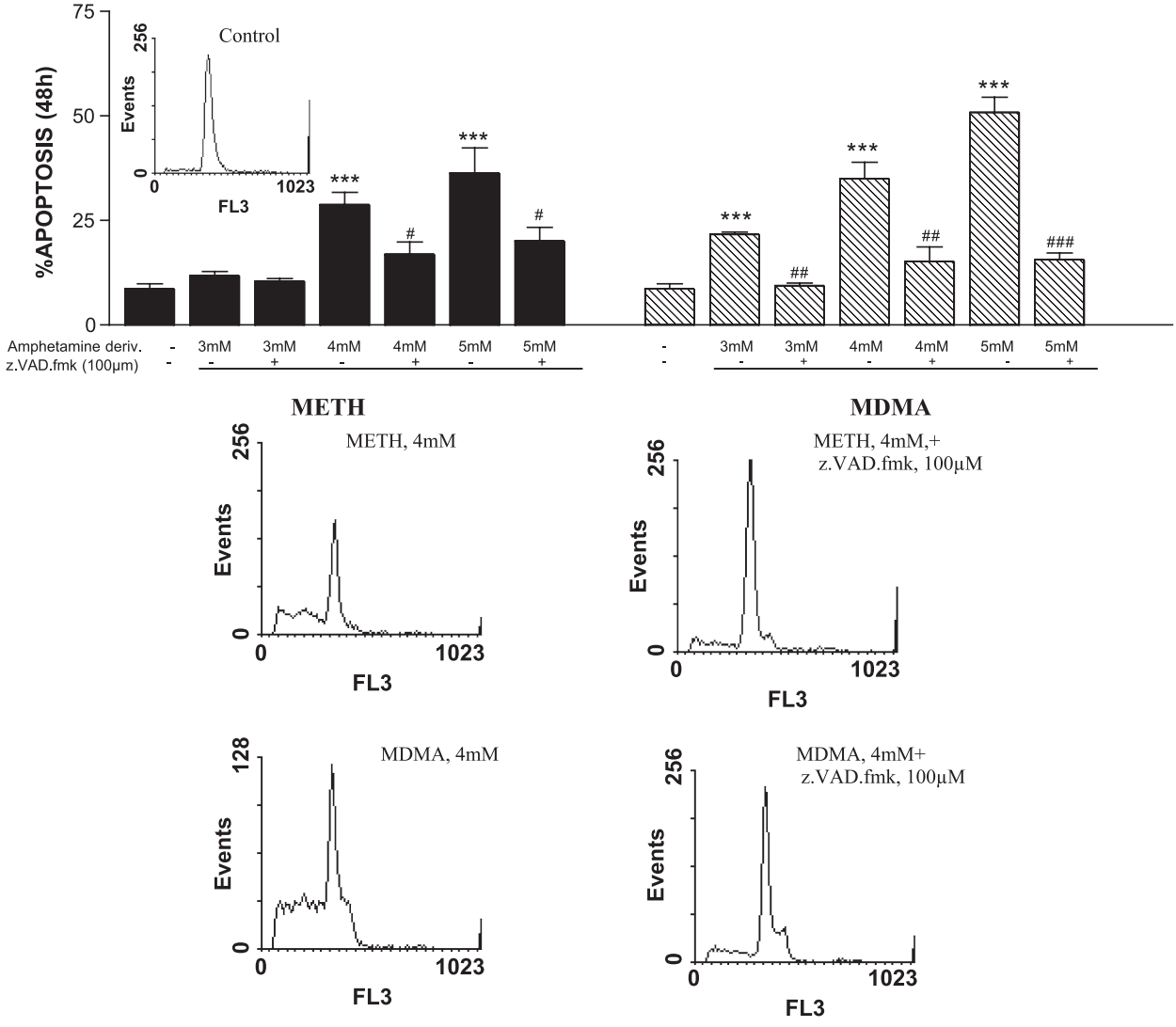

Fig. 4. Flow cytometry analysis of amphetamine derivative-induced apoptosis in CGNs shown by propidium iodide fluorescence histograms. Representative images of flow cytometric analysis of CGNs cultures after METH or MDMA 48 h treatment in absence or presence of z.VAD.fmk are showed. Bar chart shows the percentage of apoptotic cells in the different conditions tested. The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test ***$P < 0.001$ vs. control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. amphetamine derivatives.

Caspases activity. We used the colorimetric substrate Ac-DEVD-*p*-nitroaniline for the determination of caspase-3, Ac-VEID-pNA for caspase-6, and Ac-LEHD-pNA for caspase-9 according to the following method: 48 h after treatment with 4 mM amphetamine derivatives, CGNs were collected in a lysis buffer (50 mM Hepes, 100 mM NaCl, 0.1% CHAPS, 0.1 mM EDTA, pH 7.4) and 0.5 μg/μl of protein was incubated with 200 μM Ac-DEVD-*p*-nitroaniline in assay buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 0.1 mM EDTA, pH 7.4) in 96-well plates at 37 °C for 24 h. Absorbance of the cleaved product was measured at 405 nm in a microplate reader (BioRad). Results were expressed as arbitrary units of absorbance.

α-Spectrin digestion immunoblotting. For α-spectrin breakdown as a caspase-3 activity measure, Western blot analysis was made using aliquots of amphetamine derivatives-treated or control cells, containing 5 μg of protein per sample. In brief, samples were placed in sample buffer [0.5 M Tris–HCl, pH 6.8, 10% glycerol, 2% (w/v) SDS, 5% (v/v) 2-β-mercaptoethanol, 0.05% bromophenol blue] and denatured by boiling at 95–100 °C for 5 min. Samples were separated by electrophoresis on 10% acrylamide gels. Thereafter, proteins were transferred to polyvinylidene fluoride (PVDF) sheets (Immobilon-P, Millipore Corp., Bedford, MA) using a transblot apparatus (BioRad). Membranes were blocked overnight with 5% nonfat milk dissolved in TBS-T buffer (Tris 50 mM; NaCl 1.5%; Tween 20, 0.05%, pH 7.5). They were then incubated with a primary antibody against α-spectrin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). After 90 min, blots were washed thoroughly in TBS-T buffer and incubated for 1 h with a peroxidase-conjugated IgG antibody (Amersham Corp., Arlington Heights, IL). Immunoreactive protein was visualised using a chemiluminescence-based detection kit following the manufacturer protocol (ECL kit; Amersham Corp.). Routinely, protein load was monitored using phenol red staining of the blot membrane.

Cytochrome C and caspase-3 immunodetection. Immunocytochemistry assay was used to evidence Cyt C release. Cells were grown on sterile glass slides and, after stimulus, were washed twice in PBS and fixed in 4% paraformaldehyde/PBS, pH 7.4, for 1 h at room temperature. When necessary, 100 nm of MitoFluor Red 589 Dye (Molecular Probes, Inc.) was added to culture before aldehyde fixation. CGNs were pre-incubated for 30 min with PBS containing 0.3% Triton X-100 and 30% normal horse serum at room temperature. After blocking, cells were incubated with

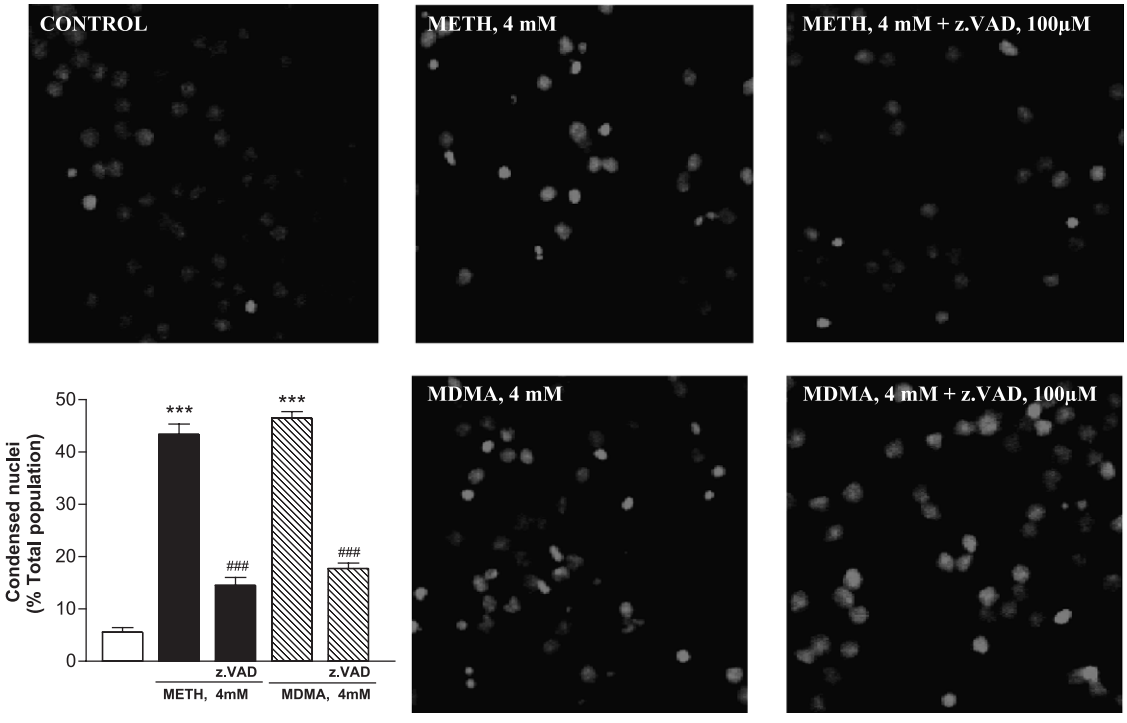

Fig. 5. METH- or MDMA-induced chromatin condensation in permeabilised CGNs after 48 h treatment in the absence or presence of z.VAD.fmk (100 μM). Following the exposure to 4 mM amphetamine derivatives, the CGNs were fixed, stained with propidium iodide, and photographed at the fluorescence microscope. For the analysis of chromatin condensation, the fluorescence microscope was used to count the nuclei. These were distinguished from the normal form the condensed nuclei using the criteria stated in Materials and methods. Values derive from counts of at least 500 nuclei in four or more random microscope fields per condition. The statistical analysis was carried out with the one-way ANOVA followed by Tukey's test; $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. amphetamine derivatives. Calibration bar, 10 μM.

monoclonal antibodies against Cyt C (1:500, Neomarkers) or caspase-3 (that recognises full-length and cleaved caspase-3, 1:1000, Cell Signaling) overnight at 4 °C. Cells were then washed extensively and incubated with secondary antibody (rhodamine-antimouse, 1:100, Boheringer) for 1 h at room temperature. Co-localisation with mitochondria was made pre-incubating cells with 0.5 μM Mito Tracker Red (CM-H_2XROS) (Molecular Probes, Inc.) for 1 h before fixative protocol. Coverslips were thoroughly washed and mounted in Mowiol 4-88 and immunosignal analysis was performed under fluorescent light using a 100× lens (Eclipse TE 200, Nikon Instech, Corp.) and their digitised images were captured.

Statistics. Data are expressed as mean ± SEM values. Statistical significance was assessed by ANOVA followed by Tukey–Kramer test. Differences were accepted as significant at $P < 0.05$. Data are expressed as percentages relative to control values. All experiments were replicated a minimum of three times.

Results

Neurotoxicity of amphetamine derivatives

Microscopic evaluation of METH and MDMA effects on CGNs revealed no morphological differences between untreated controls and amphetamine derivative-exposed cells after the first hour. The first neuritic cell damage became visible after 24 h of treatment. The neurotoxic effect was obvious within 48 h of incubation, especially with MDMA and to a lesser extent with METH. This neurotoxic effect was shown as destroyed neuronal dendrites, the presence of shrunken cells, and the qualitative reduction of viable cells (Fig. 1A). The effects of the amphetamine derivatives were underscored by quantification of neurotoxicity by using the MTT test. The exposure of culture to different concentrations of amphetamine derivatives (1–5 mM) for 24 h caused a slight neurotoxic effect in a non-concentration-dependent manner assessed by the MTT assay. When stimuli remained in the culture for 48 h, as assessed previously with microscopic evaluation, concentration curve toxicity was obtained for METH and MDMA. Maximal neurotoxicity appears at a concentration of 5 mM; at this concentration, viability decreased up to 52.6 ± 7.3% for METH and up to 36.7 ± 2.9% for MDMA ($n = 5$) (Fig. 1B). Neither (+)MK-801 (10 μM), a selective antagonist of NMDA receptors, nor NBQX (10 μM), antagonist of AMPA/kainate, prevents cell death induced by amphetamine derivatives (Fig. 2).

Additionally, new experiments were carried out to determine the effect of z.VAD.fmk on the toxicity induced by amphetamine derivatives. This pancaspase inhibitor (100 μM) partially reduced the neurotoxic effects of METH and MDMA in a statistically significant manner (Figs. 1A and 2).

Amphetamine derivatives induced ROS in CGNs

Twenty-four hours of METH (4 mM) treatment induced an increase in ROS production up to 40% measured by laser scanning cytometer. MDMA (24, 4 mM) induced a higher increase in ROS production up to 128%. In both cases, ROS increase was totally prevented in the presence of 10 μM α-tocopherol (Fig. 3).

Amphetamine derivatives induce apoptosis in CGNs

Apoptosis was evaluated by two different methods: DNA fragmentation by flow cytometry and quantification of nuclear condensation by fluorescence microscopy. Treatment with METH or MDMA for 48 h raised, in a dose-dependent way, the number of apoptotic cells measured by flow cytometry as shown in Fig. 5. When MK-801 (10 μM) and NBQX (10 μM) were co-incubated for 48 h with

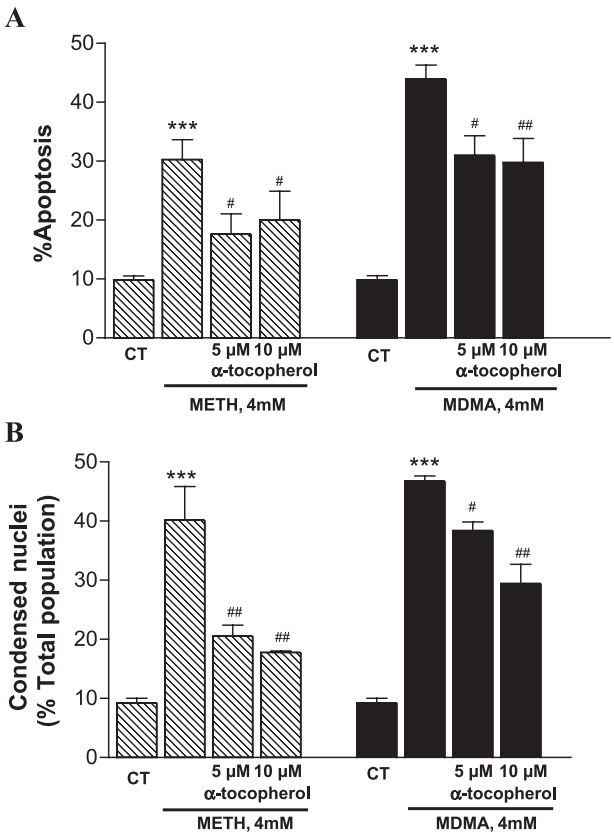

Fig. 6. Implication of radical oxygen species on the effect of amphetamine derivatives' exposure in the absence or presence of α-tocopherol. (Panel A) Apoptosis rate measured by flow cytometry. (Panel B) Chromatin condensation analysed under fluorescence light. Each point is the mean ± SEM of three to five cultures, carried out in quadruplicate. The statistical analysis used was one-way ANOVA followed by Tukey's test; ***$P < 0.001$ vs. control; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. amphetamine derivatives.

METH and MDMA (3, 4, and 5 mM), neither drug modified the percentage of apoptotic cells. In contrast, z.VAD.fmk (100 μM) reduced the percentage of the hypodiploid population in a significant way (Fig. 4). Similar results were obtained when apoptotic signals were monitored by changes in the morphology of the nuclei, after staining with PI and visualised under fluorescence. We observed an increase in the number of cells with chromatin condensation after treatment with METH and MDMA from 5.5 ± 18%, control values, to 46 ± 3.6% for METH (4 mM) and to 45.7 ± 3.53 for MDMA (4 mM). Similar results were obtained with 3 and 5 mM of both compounds tested (data not shown). Neither MK-801 (10 μM) nor NBQX (10 μM) prevented nuclear condensation after 48 h treatment with 4 mM amphetamine derivatives. However, z.VAD.fmk (100 μM) was able to prevent the nuclear condensation in a statistically significant way (Fig. 5).

α-Tocopherol attenuates amphetamine derivatives-induced neurotoxicity

The widely reported implication of oxidative stress on neurotoxicity induced by METH and MDMA prompted us to test an antioxidant compound as neuroprotective in our model. α-Tocopherol (1–15 μM) co-incubation for 48 h with 4 mM METH or MDMA increased slightly but in a statistically significant way (10–15% recovery, $P > 0.05$ vs. control values) MTT values with respect to control.

We also evaluated the effects of α-tocopherol (0.1–15 μM) on METH- and MDMA-induced apoptosis. Pretreatment with 5 or 10 μM α-tocopherol inhibited approximately 15–20% the number of cells with aneuploidic nuclei after 4 mM (48 h) METH or MDMA treatment (Fig. 6A). The quantification of the percentage of apoptotic nuclei population also showed a partial reversion with α-tocopherol in the number of pyknotic nuclei (Fig. 6B).

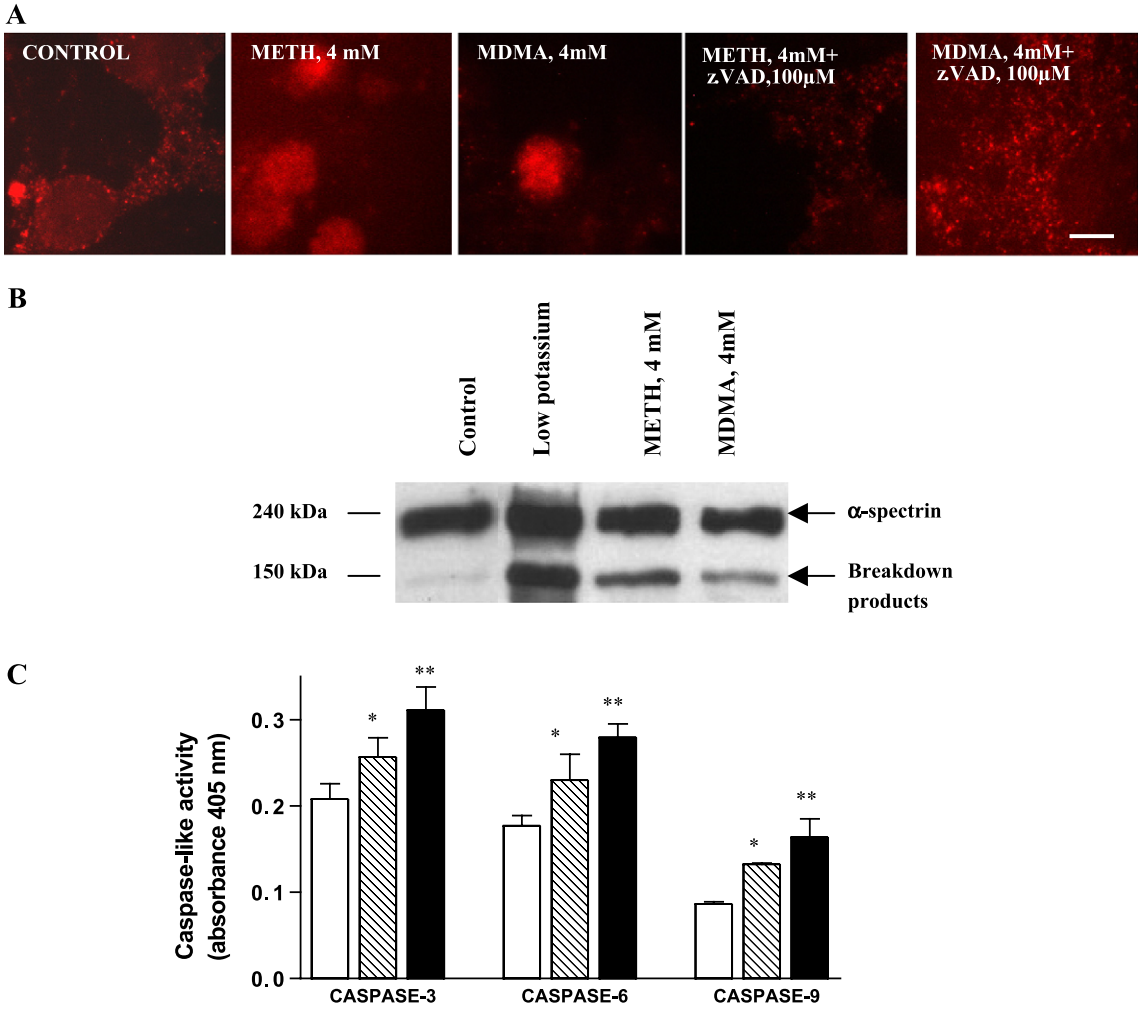

Fig. 7. (A) Immunocytochemistry against caspase-3: note the cytoplasmic staining in control and pretreated z.VAD.fmk (100 μM) cultures and the loss of staining in cytoplasma, accompanied by nuclear translocation in amphetamine derivative-treated cells and indicating the activation of caspase-3. Calibration bar, 5 μM. (B) Protein immunoblot studies of α-spectrin breakdown in the different conditions tested. (C) Bar chart shows the increase in caspase-3, -6, and -9 activity in 24-h amphetamine derivative-treated (4 mM) CGNs (open bars, control; dashed bars, METH; black bars, MDMA). Each point is the mean ± SEM of three cultures, carried out in duplicate. The statistical analysis used was one-way ANOVA followed by Tukey's test; *$P < 0.05$, **$P < 0.01$ vs. control.

Caspase-3-like pathway activation by amphetamine derivatives

We have measured the caspase-3-like enzymatic activity to further demonstrate that METH and MDMA neurotoxicity is mediated by the activation of caspase pathway. Exposure of CGNs to METH or MDMA (4 mM) for 24 h induced a significant increase (27.8 ± 0.2% and 45.3 ± 4.3%, $n = 3$) in caspase-3-like activity (Fig. 7). This activation was corroborated by immunohistochemistry using an antibody that recognises both the inactive and the cleaved form of the enzyme. We observed a nuclear translocation of the signal for caspase-3 after 48 h incubation with both of the drugs (Fig. 7). The translocation of caspase-3 from the cytoplasm to the nuclei was inhibited in the presence of z.VAD.fmk (100 μM). The caspase-9 and caspase-6 were activated in the same degree by amphetamine derivatives (Fig. 7) indicating a apoptosis pathway linked to this protease family.

Moreover, additional experiments were carried out using cleavage of α-spectrin as a measure of caspase-3 activity. α-Spectrin (280 kDa) when cleaved by caspase-3 rend a 120-kDa product that is unique in apoptotic conditions (McGinnis et al., 2003; Nath et al., 1996, 2000) as demonstrated in CGNs after 24 h potassium deprivation (Fig. 7). Our result showed an intense band in 120-kDa level, whereas in the intact α-spectrin level was decreased both by METH or MDMA, 4 mM for 24-h-treated CGNs with respect to control cultures (Fig. 7). These results corroborate immunohistochemistry results and biochemical measure of caspase-3 activation, thus demonstrating the activation of caspase-3 in amphetamine-derivatives-induced neuronal cell death in CGCs.

The correlation among the release of Cyt C, activation of caspase pathway, and subsequent neuronal apoptosis is well known. To assess whether Cyt C was released after amphetamine derivative apoptotic stimuli in CGNs, immuno-

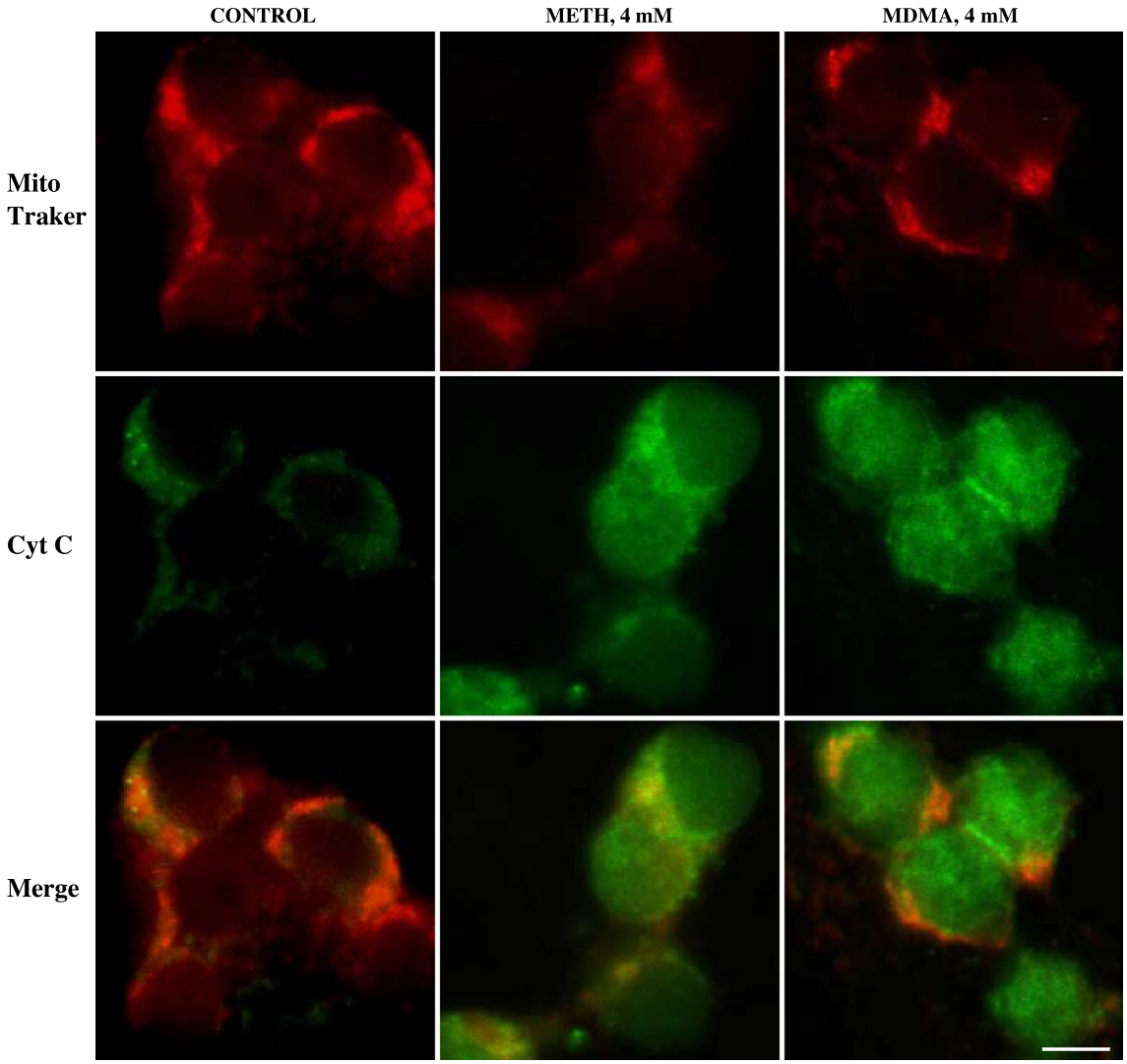

Fig. 8. Immunocytochemistry against Cyt C (green) on control cells, 48 h METH-, and MDMA-treated cultures. Co-localisation with mitochondria was carried out using Mito Tracker Red. Similar results were obtained in three different experiments. Calibration bar, 5 μM.

cytochemistry analysis was performed and co-localisation with mitochondria was made. Results revealed a marked increase in the Cyt C release from mitochondria after 24 h METH (4 mM) or MDMA (4 mM) treatment (Fig. 8).

Discussion

Our data demonstrate the neurotoxic effect of METH and MDMA and the implication of apoptotic pathways in the cell death induced by these drugs in CGNs. Despite recent advances in our understanding of the molecular events underlying cell death, the distinction between apoptosis and necrosis still depends primarily on morphological criteria (Du et al., 1997a, 1997b). Mitochondria have been implicated in both the necrotic and apoptotic cell death pathways with respect to neurodegenerative disorders (Cassarino and Bennet, 1999). A necrotic pathway is usually induced by more severe toxic insults, with ATP reserves depleted below survival levels. In vitro, a rapid onset of neurotoxicity and the absence of chromatin condensation and pyknotic nuclei indicate a necrotic death pathway. In our model, only long-lasting exposure to METH or MDMA induced a significant neurotoxicity, accompanied, according to other reports (Cubells et al., 1994), by an extensive fraction of shrunken cells with cytoplasmic vacuolisation and condensed nuclei indicating apoptotic rather than necrotic cell death. Vacuolisation may be secondary to swelling of mitochondria. Swelling can occur as a result of increased mitochondrial matrix volume, which might lead to a subsequent rupture of the outer membrane, release of Cyt C, and other proteins normally sequestered in the mitochondrial intermembrane space (Atlante et al., 1999a, 2001; Kennedy et al., 1999).

We have demonstrated that treatment with METH or MDMA increases Cyt C levels into the cytoplasma in CGNs. Moreover, METH and MDMA exposure results in a marked increase in caspase-3-like activity that was reverted in the presence of z.VAD.fmk. The implication of the caspase pathway after METH treatment has been described in a rat striatal cell line (Deng et al., 2002), and very recently, MDMA has been described as inducing Cyt C release and activation of caspase-3 in hepatic cells (Montiel-Duarte et al., 2002). However, to our knowledge, this is the first report linking the proteolytic processing and activation of a specific ICE/CED3-related cysteine protease, that is, a caspase-3-like protease, with the neurotoxic actions of MDMA. Moreover, the cell damage induced by the METH or MDMA that was assessed using MTT was suppressed by the broad-range caspase inhibitor z.VAD.fmk, indicating that challenged cells died primarily by apoptosis. Otherwise, the temporal association between the elevation of caspase-3 like protease activity and Cyt C levels in amphetamine derivatives-treated CGNs, accordingly with several neurotoxic stimulus, as MPP^+ or glutamate (Du et al., 1997a, 1997b), strongly suggests that the activation of a caspase-3-like protease plays a critical role in the apoptotic cascades induced by these compounds.

There are several mechanisms linked to the toxicity of amphetamine derivatives that can be associated with our results. First, the production of free radical species resulting in an oxidative damage (Deng and Cadet, 1999). As previously mentioned, there are several ways by which treatment with amphetamine derivatives may result in the production of ROS. The mitochondrion may be, by itself, a source of ROS through leakage from the electronic transport chain damaged by METH or MDMA. According to this, our results show that amphetamine derivatives induced ROS generation, reverted by α-tocopherol, and that neurotoxicity is significantly reduced by this antioxidant. Second, the involvement of excitotoxins as a result of mitochondrial damage could be sufficient to trigger the excitotoxic cascade, especially through glutamate release (then mediated by glutamate receptor activation) (Atabay et al., 1996; Zhang et al., 2000). However, in our model, the role of the excitotoxic cascade as a main process mediating amphetamine derivatives-induced neurotoxicity in CGNs can be rejected due to the lack of effect of MK-801 and NBQX on viability and apoptosis.

In this work, and in agreement with other authors (Ankarcrona, 1998; Kroemer et al., 1997; Murphy et al., 1999), evidence supporting a critical role for mitochondrial function in apoptosis is reported. We propose that through free radical formation, METH and MDMA promote a disruption of the mitochondrial membrane potential, assessed by MTT, and induce the release of Cyt C. As reported by other authors, these events may be common in neurones undergoing apoptosis after incubation, for instance, with glutamate or MPP^+ (Du et al., 1997a, 1997b), both of which promote apoptosis. The release of Cyt C or other apoptogenic factors into the cytoplasm following neurotoxic insult ultimately lead to activation of caspase-3-like molecules, resulting in cell death via apoptosis. Accordingly, results showed a marked increase in caspase-3-like activation for both compounds tested, measured by Western blot (α-spectrin breakdown), inmunohistochemistry against capase-3, and measuring enzymatic activity. The caspase cascade contributes to antioxidant system degradation, thus increasing ROS production and leading to programmed cell death (Budihardjo et al., 1999; Lee and Wei, 2000).

Our data suggest that, in CGNs, METH and MDMA induced cell death through an apoptotic pathway, implicating mitochondrial dysfunction with subsequent Cyt C release and caspase cascade activation. The mechanisms underlying neuronal cell death induced by illicit and recreational drugs such METH and MDMA, which cause degeneration of several regions of the brain (Deng et al., 1999; Ernst et al., 2000), allow us to speculate about the possibility that brain-penetrable inhibitors of one or more caspases could represent therapeutic tools to prevent neuronal cell death in habitual consumers of these compounds.

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