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## Research Report

# Mitochondrial dysfunction and caspase activation in rat cortical neurons treated with cocaine or amphetamine

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### ABSTRACT

Drug abuse is associated with brain dysfunction and neurodegeneration, and various recreational drugs induce apoptotic cell death. This study examined the role of the mitochondrial apoptotic pathway in psychostimulant-induced neuronal dysfunction. Using primary neuronal cultures, we observed that amphetamine ( $IC_{50} = 1.40$  mM) was more potent than cocaine ( $IC_{50} = 4.30$  mM) in inducing cell toxicity. Apoptotic cell death was further evaluated using cocaine and amphetamine concentrations that moderately decreased cell reduction capacity but did not affect plasma membrane integrity. Compared to cocaine, amphetamine highly decreased the mitochondrial membrane potential, as determined using the fluorescent probe rhodamine-123, whereas both drugs decreased mitochondrial cytochrome c. In contrast to amphetamine, cocaine cytotoxicity was partly mediated through effects on the electron transport chain, since cocaine toxicity was ameliorated in mitochondrial DNA-depleted cells lacking mitochondrially encoded electron transport chain subunits. Cocaine and amphetamine induced activation of caspases-2, -3 and -9 but did not affect activity of caspases-6 or -8. In addition, amphetamine, but not cocaine, was associated with the appearance of evident nuclear apoptotic morphology. These events were not accompanied by differences in the release of the apoptosis-inducing factor (AIF) from mitochondria. Our results demonstrate that although both amphetamine and cocaine activate the mitochondrial apoptotic pathway in cortical neurons, amphetamine is more likely to promote apoptosis.

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## 1. Introduction

Cocaine and amphetamine are commonly abused illicit drugs. These drugs increase extraneuronal dopamine, which results in stimulation of brain reward circuits. Cocaine inhibits the plasma membrane dopamine transporter (DAT), thus preventing reuptake and recycling of released dopamine, and increases the activity of the vesicular monoamine transporter (VMAT2) (Brown et al., 2001). In contrast, amphetamine inhibits the VMAT, increasing free cytosolic dopamine that subsequently accesses the synapse via reverse transport through the DAT [see Fleckenstein et al., 2000, Sulzer et al., 2005 for review].

The reward circuit is a common target for drugs of abuse (Koob, 1992). It comprises dopaminergic neurons projecting from the ventral tegmental area of the midbrain to several limbic forebrain structures: the nucleus accumbens and frontal region of the cerebral cortex. Thus, the frontal lobe is involved in establishing the persistent physiological consequences of addiction and reward-related behaviors (Rolls, 2004; Steketee, 2003; Tzschentke and Schmidt, 2000). The primary cortical targets of the mesocortical dopaminergic inputs in rats include the medial prefrontal cortex and the orbital prefrontal cortex (Kolb et al., 2004). Volkow et al. (1996) previously used positron emission tomography (PET) to demonstrate the importance of these brain regions in human addiction. Frontal lobe dysfunction mediates compulsive behavior, impulsivity, and poor decision making (Hester and Garavan, 2004; Lyvers, 2000; Stout et al., 2004), clinical features observed in human drug addicts.

In addition to the dopamine-dependent effects, the *in vivo* effects of psychostimulants, especially cocaine, on the cells of the affected structures may also be due to blockade of sodium channels, hypoglycemia, hypoxia and decrease of nutrient supply, resulting from vasoconstriction, among other mechanisms [see Olsen, 1995 for review].

Several lines of investigation suggest either necrotic or apoptotic neuronal death may underlie drug-of-abuse-related behavioral changes. Necrotic cell death involves the loss of membrane integrity and selective permeability, whereas apoptotic cell death is characterized by membrane blebbing, cell shrinkage and chromatin condensation and fragmentation. These apoptotic changes are often accompanied by caspase activation, which can occur either through extrinsic or intrinsic pathways. Extrinsic stimuli activate caspases-8 or -10, whereas intrinsic stimuli induce the release of mitochondrial proteins such as cytochrome c, which activates caspase-9. In either case, these initiator caspases ultimately activate caspase-3, an effector caspase that degrades cellular components. Caspase-2 causes mitochondrial permeabilization and increases cytochrome c release (Enoksson et al., 2004), suggesting that caspase-2 promotes mitochondrial-dependent intrinsic caspase cascades. In addition, caspase-2 was also reported to act as an effector caspase [see Zhivotovsky and Orrenius, 2005, for review]. Besides the caspase-dependent apoptotic processes, caspase-independent events may also occur [see Kroemer and Martin, 2005, for review]. One of the most studied proteins involved in caspase-independent apoptosis is the apoptosis-inducing factor (AIF), which may be

released by mitochondria and translocated to the nucleus, where it induces DNA fragmentation independently of caspases.

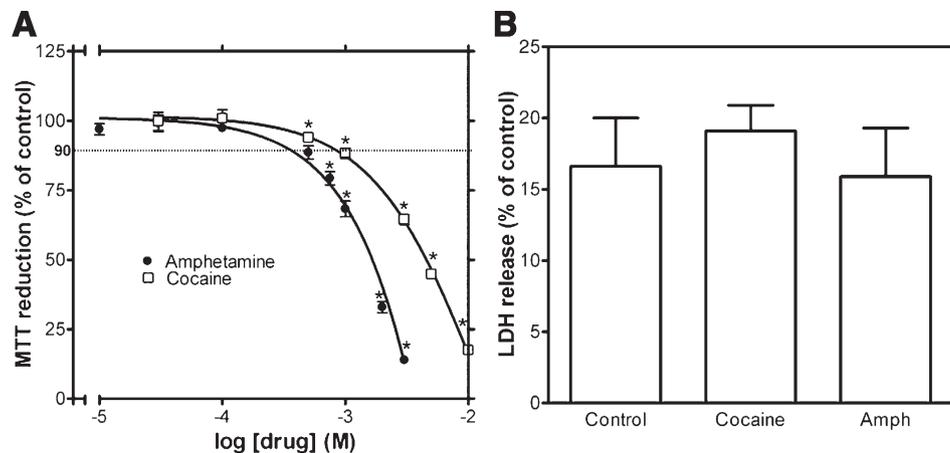
The neurotoxicity of amphetamines is well established (Cadet et al., 2003). It was previously shown that amphetamine and amphetamine derivatives can induce apoptosis in cultured neocortical neurons (Stumm et al., 1999). Cadet et al. (1997) also demonstrated methamphetamine-induced apoptosis in immortalized neural cells. Moreover, in cerebellar granule cells, amphetamine derivatives initiate caspase activation (Jimenez et al., 2004). Cocaine is also cytotoxic to several cell types, including PC12 cells (Oliveira et al., 2002), bovine coronary artery endothelial cells (He et al., 2000), rat fetal myocardial cells (Xiao et al., 2000), rat spleen cells (Pacifci et al., 2003) and canine cerebral vascular smooth muscle cells (Su et al., 2003). In mouse cortical neurons, cocaine was shown to induce apoptotic cell death (Nassogne et al., 1998), although upstream mechanisms were not clarified. Cocaine also inhibited neurite outgrowth in differentiated PC12 cells (Zachor et al., 2000). These two last reports strongly suggest that apoptotic cell death plays an important role in psychostimulant-induced loss of neuronal connectivity.

Amphetamine and cocaine interfere with mitochondrial function (Brown and Yamamoto, 2003; Yuan and Acosta, 2000). Nevertheless, the importance of mitochondrial dysfunction in psychostimulant-induced neuronal loss is unclear. In our current study, we used rat primary cortical neurons to investigate the role of the mitochondrial apoptotic pathway in cocaine and amphetamine-induced neurodegeneration. Although both drugs of abuse activate caspase cascades, amphetamine alone clearly produced nuclear fragmentation, suggesting greater overall activation of cell death pathways. Also, using mitochondrial DNA-depleted cells ( $\rho^0$  cells) previously shown to lack a functional respiratory chain (Cardoso et al., 2001), we found that amphetamine and cocaine affect mitochondrial function through different mechanisms.

## 2. Results

### 2.1. Amphetamine is more potent than cocaine at eliciting cell death

Amphetamine and cocaine neurotoxicity were evaluated by tracking dose-dependent changes in MTT reduction, a measurement of cell viability that reflects cell reducing capacity. Although both drugs induced a dose-dependent decrease in cell viability after 24-h exposure, amphetamine was more potent than cocaine, since both drugs appear to produce the same maximal effect, but the  $IC_{50}$  of amphetamine is shifted to the left ( $IC_{50}$  ~1.4 mM for amphetamine,  $IC_{50}$  ~4.3 mM for cocaine) (Fig. 1A). To avoid inducing necrotic cell death in subsequent experiments, drug concentrations corresponding to the  $IC_{10}$  values (dotted line in Fig. 1A) were identified. In this regard, we found 500  $\mu$ M amphetamine and 1 mM cocaine induced moderate but significant changes in MTT reduction. LDH leakage analysis indicated that these concentrations did not alter cell membrane integrity (Fig. 1B).



**Fig. 1** – Analysis of cell viability upon exposure to cocaine and amphetamine. **(A)** Dose-dependent changes in cell reducing capacity, determined by the MTT assay, in cortical neurons incubated with cocaine and amphetamine. Cortical neurons were incubated with increasing concentrations of cocaine (0.01–10 mM) or D-Amphetamine (0.01–3 mM), for 24 h, and the toxic effects were evaluated by the MTT reduction assay. The dotted line represents the IC<sub>10</sub> values, corresponding to 1 mM cocaine and 0.5 mM D-Amphetamine. **(B)** Analysis of membrane integrity upon exposure of cortical neurons to the drugs of abuse. Membrane integrity was determined by the LDH leakage assay, by following the rate of conversion of NADH to NAD<sup>+</sup> at 340 nm, after exposure (24 h) of cortical neurons to the concentrations of drugs of abuse close to the IC<sub>10</sub> values, determined by the MTT assay. Data are the means  $\pm$  SEM of 3–5 experiments performed in triplicate. Statistical significance: \* $P < 0.001$  as compared to the control.

## 2.2. Amphetamine and cocaine decrease mitochondrial membrane potential and mitochondrial cytochrome *c*

Because the neurophysiological consequences of amphetamine and cocaine-induced mitochondrial dysfunction are poorly characterized, we used rhodamine-123 to assess effects of these drugs on the mitochondrial membrane potential ( $\Delta\Psi_m$ ) and also evaluated mitochondrial cytochrome *c* release. These analyses were performed using rat primary neuronal cultures. We further evaluated whether cocaine and amphetamine toxicity is mediated through effects on the electron transport chain by exposing NT2 rho<sup>+</sup> and NT2 rho<sup>0</sup> cells to these drugs. NT2 rho<sup>0</sup> cells were previously shown to have trace-to-no complex I, II/III, and IV activity, but their mitochondria still maintained a  $\Delta\Psi_m$  (Cardoso et al., 2001). NT2 cells were shown to be more resistant to the drugs in comparison with cortical neurons. Therefore, concentrations of the drugs near the IC<sub>50</sub> in NT-2 rho<sup>+</sup> cells were used (Fig. 2C).

In cortical neurons, both amphetamine and cocaine induced a decrease in mitochondrial cytochrome *c*, determined as a loss of co-localization between the labeling of cytochrome *c* and MitoTracker Green (Fig. 2A). This suggests transfer of cytochrome *c* to the cytosol. A decrease in overall cytochrome *c* staining after drug incubations was observed, which may reflect loss during the washing procedure, or an inability of the antibody to detect apoptosome-sequestered cytosolic cytochrome *c*. Moreover, some denaturation of cytosolic cytochrome *c* may occur that may account for the decrease in overall staining, since the antibody is directed against the native form of cytochrome *c*.

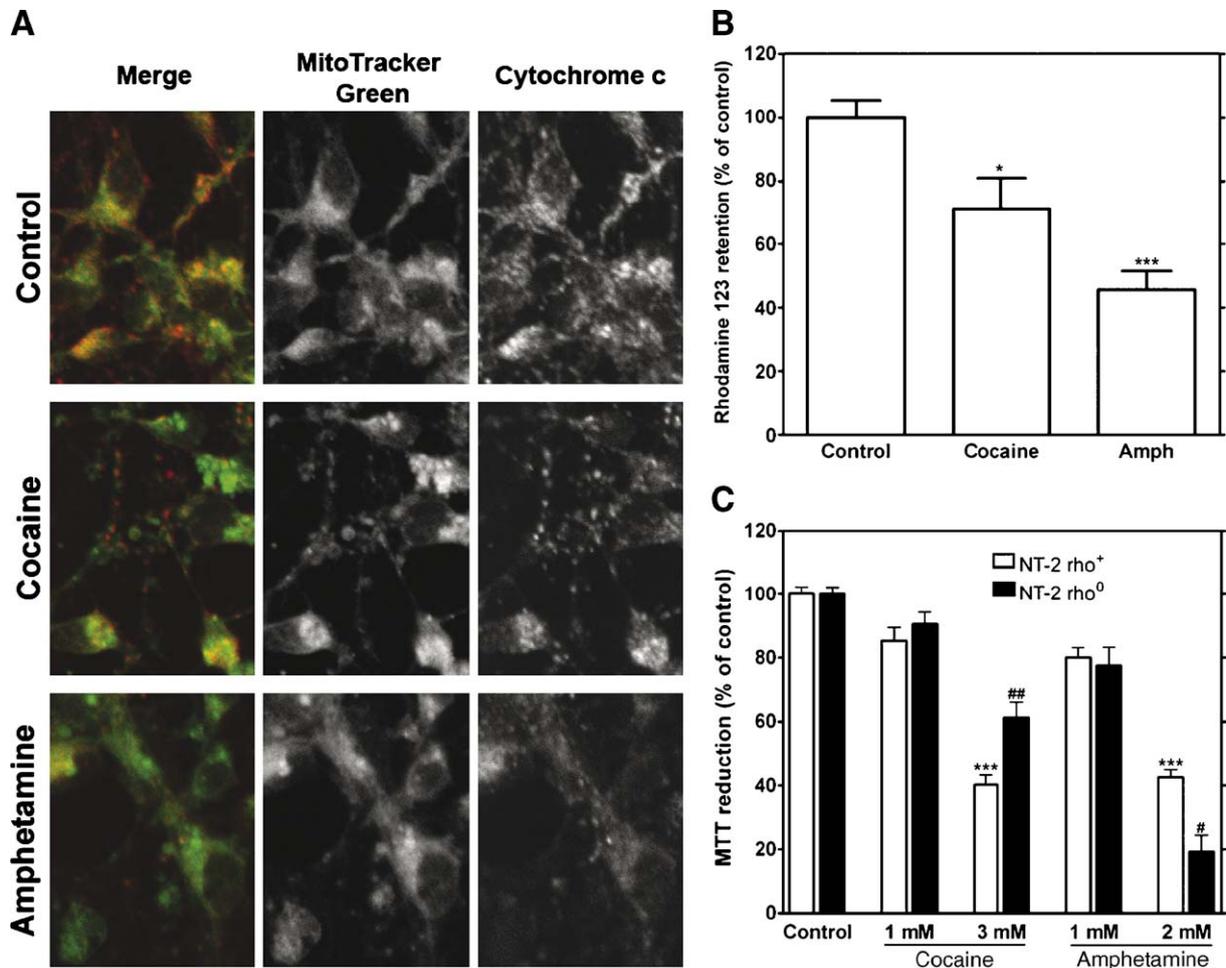
The fluorescent probe rhodamine-123 (Rh123, Fig. 2B) is retained by mitochondria as a direct function of  $\Delta\Psi_m$ . Cocaine and amphetamine significantly decreased Rh123 retention by

29% and 54.5%, respectively, suggesting these drugs induced substantial mitochondrial depolarization. Interestingly, although the drug concentrations studied in this experiment caused similar degrees of neuronal dysfunction (Fig. 1), amphetamine caused a more robust dissipation of the  $\Delta\Psi_m$  than did cocaine.

Furthermore, although cocaine altered cell viability in NT2 rho<sup>+</sup> cells nearly at its IC<sub>50</sub> concentration, its effects on NT2 rho<sup>0</sup> cells were ameliorated (Fig. 2C). This indicates cocaine's cytotoxic effects derive at least partly from interactions with the mitochondrial electron transport chain. For amphetamine, however, the toxic effects were more pronounced in rho<sup>0</sup> cells than rho<sup>+</sup> cells (Fig. 2C), suggesting the absence of a functional mitochondrial respiratory chain renders cells more sensitive to stimuli that largely reduce  $\Delta\Psi_m$ .

## 2.3. Caspase activation by amphetamine and cocaine

Since amphetamine and cocaine exposure reduced mitochondrial cytochrome *c* and  $\Delta\Psi_m$ , we further determined whether these agents also activated caspase enzymes. Both drugs induced an approximate 1.5-fold increase in caspase-9 activity (Fig. 3A). Extending this observation, we found amphetamine in particular caused a substantial increase in caspase-9 proteolytic processing (Fig. 3F). In contrast, caspase-8 was not activated by 24-h exposure to either drug (Fig. 3B). Amphetamine and cocaine induced, respectively, two- and three-fold increases in caspase-2 (Fig. 3C), previously reported to increase mitochondrial permeabilization to cytochrome *c* (Enoksson et al., 2004). Among the effector caspases, both drugs caused a three-fold increase in caspase-3 activity (Fig. 3D). We also verified this finding by Western blot analysis of pro-caspase-3 proteolytic



**Fig. 2 – Analysis of mitochondrial function. (A)** Immunocytofluorescence analysis of cytochrome *c* and the mitochondria, using MitoTracker Green, upon exposure to the drugs of abuse. Cortical neurons were incubated with cocaine (1 mM) or amphetamine (Amph, 0.5 mM), for 24 h. **(B)** Analysis of rhodamine-123 (Rh123) retention capacity of cells after exposure to the drugs of abuse. Rh123 retention capacity was used to evaluate  $\Delta\Psi_m$  in cortical neurons incubated with 1 mM cocaine or 0.5 mM amphetamine, for 24 h. The cells were incubated with 1  $\mu$ M Rh123, for 10 min. The fluorescence of the probe was then recorded before ( $F_i$ ) and after ( $F_f$ ) cell permeabilization with Triton X-100. Rh123 retention capacity was calculated by the difference:  $F_f - F_i$ . **(C)** Analysis of stimulant cytotoxicity in NT-2 rho<sup>+</sup> and rho<sup>0</sup> cells following the MTT viability assay. NT-2 cells were incubated with cocaine (1 or 3 mM) or D-Amphetamine (1 or 2 mM), for 24 h, and the toxic effects were evaluated by the MTT reduction assay. Images are representative of 3 experiments, performed in duplicate. Quantitative data are the means  $\pm$  SEM of 3–5 experiments performed in duplicate. Statistical significance: \* $P < 0.05$ , \*\*\* $P < 0.001$  compared to the control, # $P < 0.05$ , ## $P < 0.01$  compared to NT-2 rho<sup>+</sup> cells.

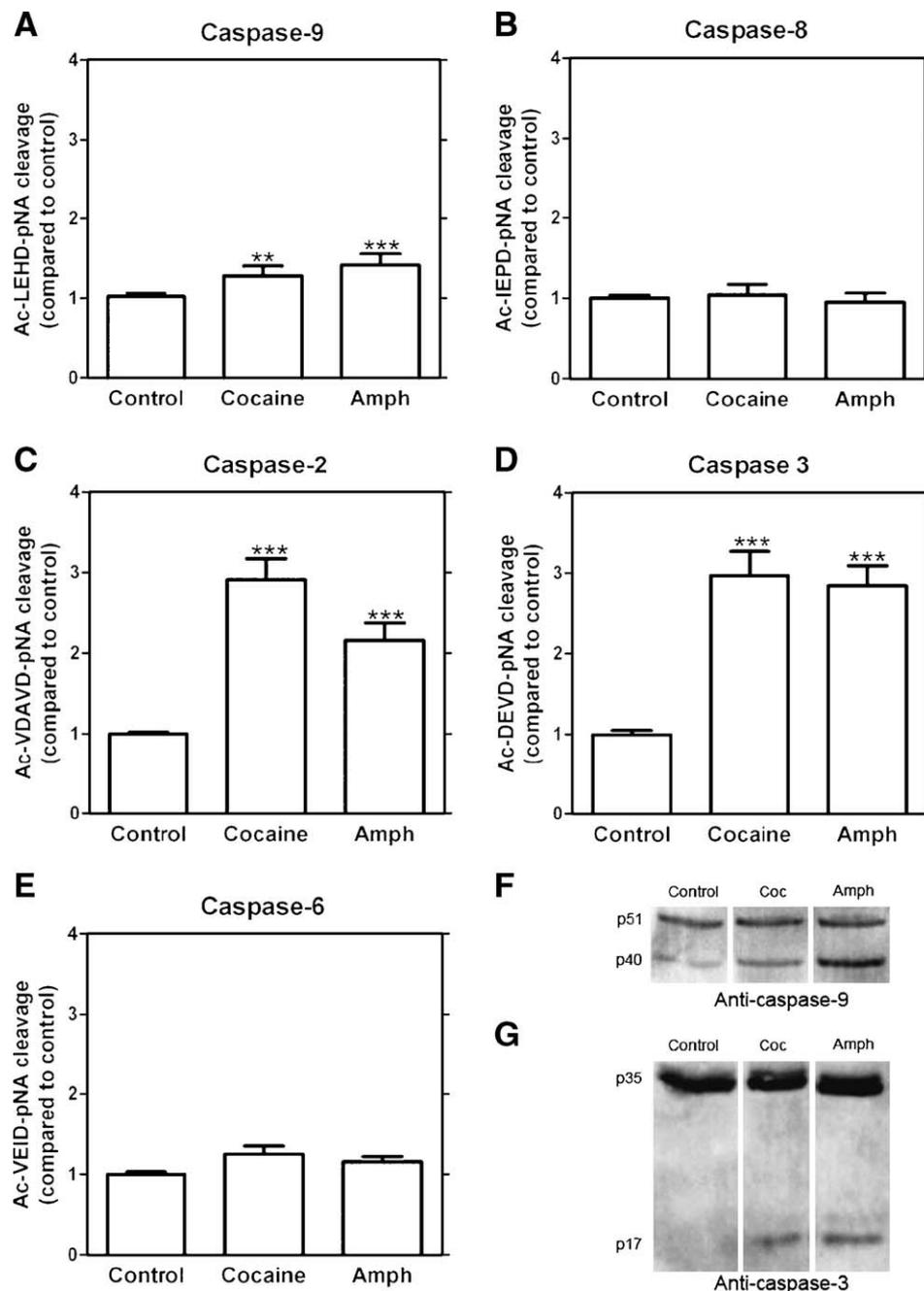
processing (Fig. 3G). Caspase-6 activity was not affected by cocaine and amphetamine (Fig. 3E).

#### 2.4. Evidence for induction of nuclear apoptotic morphology by amphetamine

We further evaluated amphetamine and cocaine-induced chromatin condensation/fragmentation using the fluorescent probes Syto-13 and PI. Although we used exposure parameters previously shown to slightly decrease MTT reduction and increase caspase-3 activity, we only observed evidence for significant DNA fragmentation with amphetamine. Specifically, amphetamine increased the number of apoptotic cells by  $15 \pm 2.5\%$  (Fig. 4). With cocaine, the number of apoptotic cells was not statistically different from

the control. The fact that amphetamine only induces a ~15% increase in DNA fragmentation (Fig. 4) although a high level of caspase activation occurs (Fig. 3) may be explained by the fact that these events correspond to different time points of apoptotic cell death. In the same context, cocaine induced a small, but significant, decrease in cell reduction capacity at the same time point where no DNA fragmentation (Fig. 4) or membrane disruption (Fig. 1B) occur. This decrease in cell reduction capacity may reflect early changes in metabolic activity, namely due to inhibition of the mitochondrial respiratory chain induced by cocaine and may not immediately correspond to cell death.

In addition, PI labeling was observed in neither drug-treated nor untreated cells, indicating that our exposure conditions did not alter plasma membrane integrity and

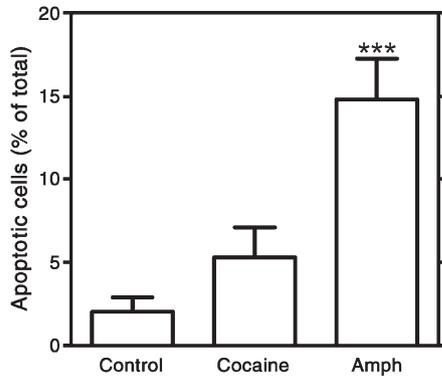


**Fig. 3** – Caspases activation in cortical neurons incubated with the drugs of abuse. The figure shows the activity of initiator (A–C) or effector (D–E) caspases, determined by the extent of cleavage of caspases substrates: Ac-LEHD-pNA (A), Ac-IEPD-pNA (B), Ac-VDVAD-pNA (C), Ac-DEVD-pNA (D) or Ac-VEID-pNA (E), tested to evaluate the activity of caspases-9, -8, -2, -3 and -6, respectively. The cells were incubated with 1 mM cocaine or 0.5 mM amphetamine, for 24 h. The processing of pro-caspases-9 (F) and -3 (G) was also examined by Western blotting. Data are the means  $\pm$  SEM of 3–5 experiments performed in duplicate. Statistical significance: \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to the control.

therefore did not cause necrotic cell death. This finding was consistent with LDH release data (Fig. 1B). Therefore, both amphetamine and cocaine, when used in concentrations that do not elicit necrotic cell death, can still activate mitochondria-initiated programmed cell death pathways. However, despite similar patterns and magnitudes of caspase activation (Fig. 3), amphetamine is more likely to cause cell death by apoptosis.

#### 2.5. AIF translocation does not explain increased DNA fragmentation induced by amphetamine

Since amphetamine and cocaine induced similar levels of caspase activation but different levels of apoptotic morphology, we quantified the mitochondrial AIF content (Fig. 5A) and analyzed the subcellular localization of this protein (Fig. 5B). AIF is a mitochondrial protein that may be translocated to the



**Fig. 4 – Analysis of DNA condensation/fragmentation in cortical neurons exposed to the drugs of abuse. Syto-13 and propidium iodide (PI) were used to evaluate apoptotic cell death upon exposure to cocaine (1 mM) or amphetamine (0.5 mM), for 24 h. After 3 min of loading with the fluorescent probes, the cells were immediately visualized in a fluorescence microscope. About 500–700 cells were counted per coverslip. Data are the means  $\pm$  SEM of 3–4 experiments performed in duplicate coverslips. Statistical significance: \*\*\* $P < 0.001$  compared to the control.**

nucleus and induce nuclear fragmentation independently of caspase activation.

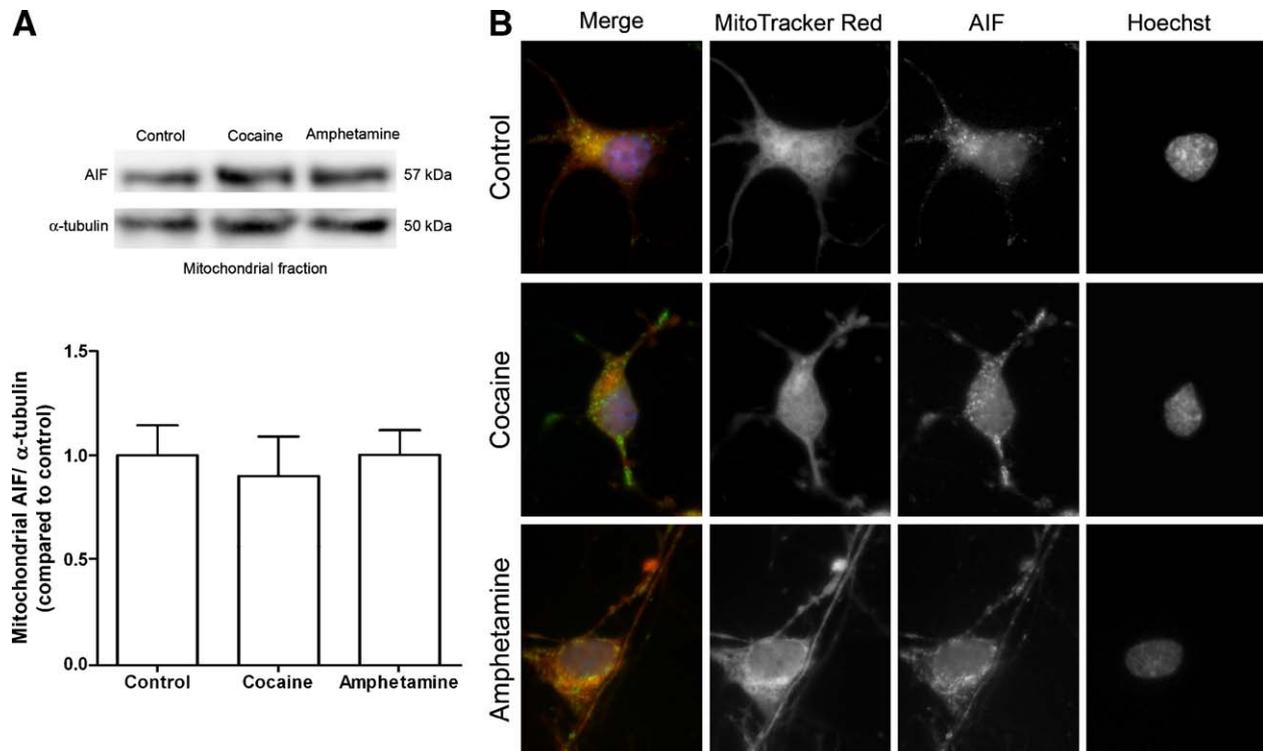
We observed that mitochondrial AIF content was not altered by cocaine or amphetamine (Fig. 5A). In accordance, the subcellular localization of AIF was maintained in the

majority of the cells (Fig. 5B) after incubation with the drugs, when compared to control conditions.

### 3. Discussion

Cocaine and amphetamine activate the mitochondrial apoptotic pathway in rat primary cortical neuron cultures. Both drugs decrease mitochondrial cytochrome c content and activate caspases-9 and -3. However, with equivalent induction of caspase enzymes, amphetamine is more likely to cause apoptosis than cocaine. The mitochondrial respiratory chain appears to play a role in this toxicity differential. Cocaine toxicity, but not amphetamine toxicity, is at least partly mediated through interactions with the electron transport chain, as cocaine is less toxic to  $\rho^0$  than  $\rho^+$  cells. In contrast, amphetamine toxicity is enhanced in  $\rho^0$  cells not possessing a functional mitochondrial respiratory chain, reflecting an increased sensitivity of  $\rho^0$  cells to stimuli that highly affect  $\Delta\psi_m$ .

Previously, we showed that cocaine (0.3 mM) and amphetamine (0.3 mM) induced the mitochondrial apoptotic pathway in the catecholaminergic PC12 cell line (Oliveira et al., 2003). Cocaine was also reported to affect mitochondrial function and induce apoptosis in bovine coronary artery cells (He et al., 2000), rat myocardial cells (Xiao et al., 2000) and rat testes (Li et al., 2003) at respective concentrations of 0.1 mM, 0.1–0.3 mM or a 15 mg/kg dose. Also, in in vitro experiments with amphetamine or its derivatives, apoptosis was observed in both rat cerebellar granule cells (Jimenez et al., 2004) and a rat striatal



**Fig. 5 – Analysis of AIF in cortical neurons. (A) Mitochondrial AIF content was analyzed by Western blotting. (B) Subcellular distribution of AIF was analyzed by immunocytochemistry using an antibody against AIF (green), the fluorescent mitochondrial marker MitoTracker Red (red) and the fluorescent nuclear marker Hoechst 33342 (blue).**

cell line (Deng et al., 2002) at concentrations of 4 mM and 2 mM, respectively. In one *in vivo* study, amphetamine at 40 mg/kg induced apoptosis in striatal GABAergic neurons through activation of the mitochondrial caspase cascade (Jayanthi et al., 2004). Although these studies indicate that psychostimulant drugs activate the mitochondrial intrinsic caspase cascade and cause mitochondria-dependent apoptosis, until now data extending these phenomena to cortical neurons were unavailable, and the involvement of the mitochondrial respiratory chain was not evaluated.

It is worthwhile to note that amphetamine and cocaine concentrations in this study were comparable to those previously used by other investigators using different cell types (Cadet et al., 1997; Deng et al., 2002; Nassogne et al., 1998; Stumm et al., 1999; Yuan and Acosta, 2000; Zaragoza et al., 2001). Moreover, cocaine plasma concentrations in human drug abusers reportedly range between 0.3  $\mu$ M and 1 mM (Yuan and Acosta, 2000) and are therefore more or less comparable to the concentrations used in this study. The cocaine used in our study contained only a small percentage (less than 4%) of benzoylecgonine. In a previous study, the cocaine metabolites ecgonine, methylecgonine and benzoylecgonine did not appear to affect neuronal viability, as determined by MAP2 labeling (Nassogne et al., 1998). We therefore believe that cocaine, rather than benzoylecgonine, mediated mitochondrial dysfunction and toxicity in our primary cortical neuron cultures. In contrast to cocaine, amphetamine is metabolized in much lower extent. So, toxicity of amphetamine metabolites is not predicted. Moreover, amphetamine used in this study was 98% pure. Therefore, impurity interference is not expected.

Our finding that amphetamine and cocaine depolarize cortical neuron mitochondria corroborates and extends previous observations from other cell types that these drugs induce mitochondrial dysfunction. The mechanisms via which these drugs induce mitochondrial dysfunction are probably not identical, as their effects on neuronal-like rho<sup>0</sup> cells are quite different. In particular, cocaine toxicity requires a functional mitochondrial respiratory chain. However, we cannot say whether this latter finding will replicate in cortical neurons.

Multiple investigations suggest cocaine influences mitochondrial function. In myocardial cells, cocaine (1 mM) inhibited complex I of the mitochondrial respiratory chain (Yuan and Acosta, 2000). Cocaine (20 mg/kg) downregulated mitochondrial gene expression in rat cingulate cortex (Dietrich et al., 2004). *In vivo* cocaine administration (25 mg/kg) decreased state 3 respiration and the respiratory control ratio (RCR) of hepatic mitochondria and decreased activity of complexes I, II/III and IV (Devi and Chan, 1997). Cocaine (1 mM) decreased mitochondrial membrane potential and ATP levels in cardiomyocytes (Xiao et al., 2000). Further, microarray data suggest mitochondrial function and energy metabolism are affected in brains of human cocaine abusers (Lehmann et al., 2003).

Amphetamine toxicity to dopaminergic systems is well known, and this toxicity may involve oxidative stress (Brown and Yamamoto, 2003; Cadet and Brannock, 1998). In dopaminergic neurons, amphetamine derivatives may especially affect complex IV function (Burrows et al., 2000). Our

current data suggest amphetamine toxicity is nevertheless not directly mediated through effects on the electron transport chain, since amphetamine toxicity was not reduced (and was actually increased) in NT2 rho<sup>0</sup> cells. Rather, our data suggest that amphetamine-induced electron transport chain alterations most likely represent a compensatory response. One amphetamine toxicity hypothesis argues that accumulation of positively charged amphetamine molecules within negatively charged mitochondrial matrices may dissipate  $\Delta\psi_m$  (Davidson et al., 2001). It was previously shown that the weak bases amphetamine and cocaine, in pharmacologically active concentrations, lead to alkalinization of intracellular organelles (Sulzer and Rayport, 1990). This model can account for the loss of mitochondrial potential induced by amphetamine and cocaine, as well as the enhanced toxicity of amphetamine within rho<sup>0</sup> cells. Further evidence in support of this mechanism comes from our recent analysis of dopamine-depleted PC12 cells, which were as susceptible to amphetamine toxicity as non dopamine-depleted PC12 cells (Cunha-Oliveira et al., 2006). Dopamine, therefore, may not be crucial for amphetamine cytotoxicity. Consistent with this, although amphetamine neurotoxicity generally manifests within monoaminergic systems (Cadet et al., 2003), non-dopaminergic neurons also show evidence of toxicity (Eisch and Marshall, 1998; Jimenez et al., 2004; Krasnova et al., 2005).

Our primary neuronal cultures were derived from rat frontal cortex. Although the frontal cortex receives dopaminergic input from the mesocortical dopaminergic pathway, there were few or no tyrosine-hydroxylase positive cell bodies present in our neuronal cultures (data not shown). In this context, Eisch and Marshall (1998) previously found methamphetamine-induced cortical neuronal damage to non-monoaminergic neuronal populations does not necessarily arise as a secondary consequence of damage to networked striatal dopaminergic neurons.

In accordance with the present results, Dietrich et al. (2005) recently demonstrated that although cocaine and amphetamine are deleterious to the brain, the adverse consequences of amphetamine exposure are more pronounced. The fact that amphetamine induced more apoptosis than a cocaine concentration causing a similar degree of caspase activation suggests amphetamine may have also uniquely activated caspase-independent cell death pathways. Interestingly, Jayanthi et al. (2004) recently found that mouse striatum methamphetamine injection triggers both caspase-dependent and -independent cell death pathways. However, in this study, AIF did not mediate this effect, since there were no changes in its subcellular localization. Several other caspase-independent pathways have been described, that could account for this effect [see Kroemer and Martin, 2005, for review].

In summary, we found both cocaine and amphetamine induce mitochondrial dysfunction (evident as a decrease in  $\Delta\psi_m$ , particularly upon exposure to amphetamine) and activate the mitochondria-dependent intrinsic caspase cascade. Nevertheless, at concentrations of drugs that do not cause necrosis, apoptosis was more evident in amphetamine-treated cells. Our data suggest that the mechanisms through which these drugs dissipate  $\Delta\psi_m$  may differ. Such mechanistic

differences, in conjunction with the ability of a particular cell to tolerate  $\Delta\psi_m$  dissipation, may influence neuronal susceptibility to cocaine and amphetamine.

## 4. Experimental procedures

### 4.1. Materials

Optimem medium, Neurobasal medium and B27 supplement were supplied by Gibco (Paisley, UK). Chymostatin, leupeptin, antipain, pepstatin A, *N*-acetyl-Ile-Glu-Pro-Asp-*p*-nitroaniline (Ac-IEPD-*p*NA), trypsin, Soybean Trypsin Inhibitor (SBTI), mouse anti- $\alpha$ -tubulin and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were supplied by Sigma Chemical Co (St. Louis, MO, USA) and *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-*p*NA) was supplied by Calbiochem (Darmstadt, Germany). NT2 cells (a neuroteratocarcinoma cell line) were obtained from Stratagene (La Jolla, CA, USA). The antibodies anti-caspase-3 and anti-caspase-9 were supplied by Cell Signaling (Beverly, MA, USA). The antibody against AIF was obtained from Santa Cruz Biotechnology Int (Santa Cruz, CA, USA). ECF and anti-mouse IgG and anti-rabbit IgG were obtained from Amersham Biosciences (Piscataway, NJ, USA); Syto-13, MitoTracker Green, MitoTracker Red, Hoechst 33342, Alexa anti-mouse IgG 488, Alexa anti-mouse IgG 594, rhodamine-123 and propidium iodide were supplied by Molecular Probes (Eugene, OR, USA); *N*-acetyl-Val-Asp-Val-Ala-Asp-*p*-nitroaniline (Ac-VDVAD-*p*NA), *N*-acetyl-Val-Glu-Ile-Asp-*p*-nitroaniline (Ac-VEID-*p*NA) and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroaniline (Ac-LEHD-*p*NA) were purchased from Biosource International, Inc. (Nivelles, Belgium). Anti-cytochrome *c* was obtained from PharMingen (San Diego, CA, USA). *D*-Amphetamine was purchased from Merck (Darmstadt, Germany). Cocaine was obtained from the “Instituto da Droga e da Toxicodependência” (IDT, Lisbon, Portugal). All other reagents were of analytical grade.

### 4.2. Cocaine analysis

A sample of seizure cocaine was initially studied in order to establish its identity and purity. The study was undertaken using NMR, EM, HPLC and GC-MS procedures according to those described in the literature (Airaksinen et al., 1999; Bujan et al., 2001; de Toledo et al., 2003). All these studies enabled the identification of cocaine and benzoylecgonine in the sample. By quantification analysis, we determined that seizure cocaine has a purity of 96%, containing a small percentage (<4%) of its metabolite benzoylecgonine.

### 4.3. Culture of cortical neurons

The frontal cortices of rat embryos (16–17 days) were dissected and the cells washed with isolation medium (in mM: 120.9 NaCl, 4.83 KCl, 1.22  $\text{KH}_2\text{PO}_4$ , 25.5  $\text{NaHCO}_3$ , 13.0 glucose, 10.0 HEPES) containing 0.3% BSA. The cells were sedimented at 1000 rpm and dissociated with isolation medium, supplemented with 0.02% trypsin and 0.04 mg/ml DNase for 10 min at 37 °C. Trypsin was inactivated by adding 0.075% SBTI (trypsin inhibitor, Sigma), and the cells

were centrifuged at 1000 rpm for 5 min. The cells were then mechanically dissociated in isolation medium containing 0.012% SBTI and then centrifuged at 1000 rpm for 5 min. The resulting pellet was resuspended in Neurobasal medium with 2% B27 supplement, 0.2 mM glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin (in 5%  $\text{CO}_2$ /95% air) and plated on poly-L-lysine-coated multiwells or coverslips. Cortical cultures contained a small percentage of glial cells (less than 10%) as assessed by immunofluorescence using anti-microtubule-associated protein (MAP2) and anti-glial fibrillary acidic protein (GFAP, not shown). After 6 days in culture, the cells were incubated with the drugs of abuse, amphetamine (10  $\mu\text{M}$ –3 mM) and cocaine (10  $\mu\text{M}$ –10 mM), for 24 h.

### 4.4. Culture of NT2 cells

Stock cultures of NT2 (a clonal human neuroteratocarcinoma cell line)  $\text{rho}^+$  cells were purchased from Stratagene (La Jolla, CA, USA). The development of the NT2  $\text{rho}^0$  cell line used in these experiments was previously described (Swerdlow et al., 1997). Cells were grown routinely in 75- $\text{cm}^2$  tissue culture flasks in Optimem Medium, supplemented with 10% heat-inactivated fetal calf serum, penicillin (50 U/ml) and streptomycin (50  $\mu\text{g}/\text{ml}$ ). Uridine (50  $\mu\text{g}/\text{ml}$ ) and pyruvate (200  $\mu\text{g}/\text{ml}$ ) were also added to  $\text{rho}^0$  cell growth medium. Both cell lines were grown and maintained at 37 °C in a humidified incubator containing 95% air and 5%  $\text{CO}_2$ . The cells were plated at  $2.5 \times 10^4 \text{ cm}^{-2}$  for cell viability assay, and incubated for 24 h with amphetamine (1–2 mM) or cocaine (1–3 mM).

### 4.5. Cell reduction capacity assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay at 570 nm (Mosmann, 1983). The cells were incubated with 0.5 mg/ml MTT in  $\text{Na}^+$  medium (in mM: 140 NaCl, 5 KCl, 1  $\text{MgCl}_2$ , 1  $\text{NaH}_2\text{PO}_4$ , 1.5  $\text{CaCl}_2$ , 5.6 glucose, 20 HEPES, pH 7.4) for 2 h and the precipitated salt was dissolved with 0.04 M HCl in isopropanol. The capacity of treated cells to reduce the tetrazolium salt was expressed as a percentage of absorbance of the control cells at 570 nm.

### 4.6. Membrane leakage assay

The integrity of the plasma membrane of cortical neurons was determined by monitoring the cell lactate dehydrogenase (LDH) leakage, which was accomplished by following the rate of conversion of NADH to  $\text{NAD}^+$  at 340 nm (Bergmeyer and Bernt, 1974). LDH release into the extracellular medium was expressed as a percentage of total LDH.

### 4.7. Immunocytofluorescence

The cells were washed with saline buffer (PBS, in mM: 137 NaCl, 2.7 KCl, 1.4  $\text{K}_2\text{HPO}_4$ , 4.3  $\text{KH}_2\text{PO}_4$  at pH 7.4) and incubated with MitoTracker Green (1  $\mu\text{M}$ ) or MitoTracker Red (750 nM) for 1 h. Then, the cells were fixed in 4% paraformaldehyde containing 4% sucrose in PBS.

#### 4.7.1. Cytochrome *c* staining

The cells were washed and incubated with 20 mM glycine. After cell permeabilization in the presence of saponin (0.1%), the cells were incubated with a specific antibody against the native form of cytochrome *c* (1:100, PharMingen). Cytochrome *c* was detected by using Alexa Fluor 594 anti-mouse IgG (1:200) as secondary antibody. The cells were visualized using a confocal microscope (BioRad MRC 600).

#### 4.7.2. AIF staining

The cells were permeabilized in 0.2% Triton X-100, for 2 min, and blocked in 3% BSA for 30 min. AIF was detected by using a specific primary antibody (1:50 in 3% BSA) and Alexa anti-rabbit 488 (1:200 in 3% BSA) as secondary antibody. The nuclei were stained by using Hoechst 33342 (1 µg/ml). The cells were visualized using an epifluorescence microscope (Zeiss Axioscope).

#### 4.8. Fluorimetric evaluation of rhodamine-123 cellular retention

The cells were loaded with 1 µM rhodamine-123 for 10 min, in the dark, at 37 °C. The fluorescence (λ<sub>ex</sub> 505 nm and λ<sub>em</sub> 525 nm) was recorded for 10 min, before and after permeabilization with 0.5% Triton X-100, in a SPEX Fluorometer spectrometer equipped with a thermostatic water bath. Rhodamine-123 retention was determined by the difference between total fluorescence (after permeabilization) and the initial value of fluorescence (Palmeira et al., 1996). Because positively charged rhodamine-123 is retained by functional mitochondria with a high mitochondrial membrane potential ( $\Delta\Psi_m$ ), a decrease of cellular retention of rhodamine-123 has been associated with a decrease in  $\Delta\Psi_m$ .

#### 4.9. Colorimetric evaluation of caspase-like activity

The cells were lysed in lysis buffer [in mM: 25 HEPES, 2 MgCl<sub>2</sub>, 1 EDTA, 1 EGTA, 2 DTT, 0.1 PMSF and 1:1000 of protease inhibitor cocktail (chymostatin, leupeptin, antipain and pepstatin A, 1 mg/ml)]. The resulting extracts were frozen and thawed three times and further centrifuged at 15,000 × *g* for 10 min (4 °C), and the protein (supernatant fraction) was quantified by the Sedmak method (Sedmak and Grossberg, 1977). The supernatant was tested for caspase-2, -3, -6, -8 and -9 activities, at 405 nm, after reaction with the substrates Ac-VDVAD-pNA, Ac-DEVD-pNA, Ac-VEID-pNA, Ac-IEPD-pNA and Ac-LEHD-pNA, respectively (100 µM, for 2 h at 37 °C). The results were normalized over the control absorbance value, using the same amount of protein (25–40 µg/assay).

#### 4.10. Western blot

##### 4.10.1. Analysis of caspases-3 and -9 activation

For analysis of caspase activation by Western blotting, the cells were treated as described above for the caspase activity assays. Proteins (30 µg/sample) were denatured and separated by SDS-PAGE (12%) and then transferred to a PVDF membrane that was then incubated with rabbit anti-caspase-3, rabbit anti-caspase-9 antibodies (1:1000). An anti-rabbit alkaline-phosphatase-bound antibody (1:25000) was used as secondary

antibody. Blots were developed using an enhanced chemi-fluorescence reagent (ECF, Amersham Biosciences). Caspases-3 and -9 blots were visualized using a STORM imaging system (Molecular Dynamics).

##### 4.10.2. Analysis of mitochondrial AIF content

Mitochondrial fractions were isolated by differential centrifugation. Briefly, cell lysates were homogenized in sucrose buffer (in mM: 250 sucrose; 20 HEPES, 10 KCl; 1.5 MgCl<sub>2</sub>; 1 EDTA; 1 DTT; 0.1 PMSF) supplemented with 1 µg/ml of protease inhibitor cocktail. The mitochondrial fraction (P2) was separated by centrifugation at 500×*g* for 12 min (4 °C) followed by centrifugation of the resulting supernatant at 12,000×*g* for 20 min (4 °C). Proteins (50 µg/sample) were denatured and separated by SDS-PAGE (10%) and then transferred to a PVDF membrane that was then incubated with mouse anti-AIF (1:500) and mouse anti- $\alpha$ -tubulin (1:20000). An anti-mouse alkaline-phosphatase-bound antibody (1:25000) was used as secondary antibody. Blots were developed using an enhanced chemifluorescence reagent (ECF, Amersham Biosciences). AIF blots were visualized using a VersaDoc imaging system (Bio-Rad) and quantified using Quantity-One software (Bio-Rad).

#### 4.11. Analysis of DNA fragmentation

Syto-13 and propidium iodide (PI) were used to evaluate apoptotic cell death following exposure to cocaine or amphetamine for 24 h. The cells, cultured on coverslips, were incubated with Syto-13 (4 µM) and propidium iodide (PI, 4 µg/ml) in Na<sup>+</sup> medium. After loading with the fluorescent probes (for 3 min), the cells were immediately visualized using a Nikon diaphot fluorescence microscope equipped with a Xenon lamp (Nikon XPS-100) and a triple filter (Omega Optical-FX 64). Apoptotic cells were identified morphologically. Approximately 500–700 cells were counted per coverslip.

#### 4.12. Statistical analysis

Data are the means ± SEM from at least three independent experiments performed in duplicate or triplicate. Statistical analysis was performed by one-way ANOVA and Bonferroni's post hoc test (*P* < 0.05 was considered significant).

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## REFERENCES

- Airaksinen, A.J., Tuppurainen, K.A., Lotjonen, S.E., Niemitz, M., Yu, M.X., Vepsalainen, J.J., Laatikainen, R., Hiltunen, J., Bergstrom, K.A., 1999. Nuclear magnetic resonance and molecular orbital study of some cocaine analogues. *Tetrahedron* 55, 10537–10546.

- Bergmeyer, H.U., Bernt, E., 1974. Lactate dehydrogenase UV assay with pyruvate and NADH. *Methods Enzymatic Anal.* 2, 574–579.
- Brown, J.M., Yamamoto, B.K., 2003. Effects of amphetamines on mitochondrial function: role of free radicals and oxidative stress. *Pharmacol. Ther.* 99, 45–53.
- Brown, J.M., Hanson, G.R., Fleckenstein, A.E., 2001. Regulation of the vesicular monoamine transporter-2: a novel mechanism for cocaine and other psychostimulants. *J. Pharmacol. Exp. Ther.* 296, 762–767.
- Bujan, L., Fernandez, P., Lafuente, N., Aldonza, M., Bermejo, A.M., 2001. Comparison of two chromatographic methods for the determination of cocaine and its metabolites in blood and urine. *Anal. Lett.* 34, 2263–2275.
- Burrows, K.B., Gudelsky, G., Yamamoto, B.K., 2000. Rapid and transient inhibition of mitochondrial function following methamphetamine or 3,4-methylenedioxymethamphetamine administration. *Eur. J. Pharmacol.* 398, 11–18.
- Cadet, J.L., Brannock, C., 1998. Free radicals and the pathobiology of brain dopamine systems. *Neurochem. Int.* 32, 117–131.
- Cadet, J.L., Ordonez, S.V., Ordonez, J.V., 1997. Methamphetamine induces apoptosis in immortalized neural cells: protection by the proto-oncogene, bcl-2. *Synapse* 25, 176–184.
- Cadet, J.L., Jayanthi, S., Deng, X., 2003. Speed kills: cellular and molecular bases of methamphetamine-induced nerve terminal degeneration and neuronal apoptosis. *FASEB J.* 17, 1775–1788.
- Cardoso, S.M., Santos, S., Swerdlow, R.H., Oliveira, C.R., 2001. Functional mitochondria are required for amyloid beta-mediated neurotoxicity. *FASEB J.* 15, 1439–1441.
- Cunha-Oliveira, T., Rego, A.C., Morgadinho, M.T., Macedo, T., Oliveira, C.R., 2006. Differential cytotoxic responses of PC12 cells chronically exposed to psychostimulants or to hydrogen peroxide. *Toxicology* 217, 54–62.
- Davidson, C., Gow, A.J., Lee, T.H., Ellinwood, E.H., 2001. Methamphetamine neurotoxicity: necrotic and apoptotic mechanisms and relevance to human abuse and treatment. *Brain Res. Brain Res. Rev.* 36, 1–22.
- Deng, X., Cai, N.S., McCoy, M.T., Chen, W., Trush, M.A., Cadet, J.L., 2002. Methamphetamine induces apoptosis in an immortalized rat striatal cell line by activating the mitochondrial cell death pathway. *Neuropharmacology* 42, 837–845.
- de Toledo, F.C.P., Yonamine, M., Moreau, R.L.D., Silva, O.A., 2003. Determination of cocaine, benzoylecgonine and cocaethylene in human hair by solid-phase microextraction and gas chromatography-mass spectrometry. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 798, 361–365.
- Devi, B.G., Chan, A.W., 1997. Impairment of mitochondrial respiration and electron transport chain enzymes during cocaine-induced hepatic injury. *Life Sci.* 60, 849–855.
- Dietrich, J.B., Poirier, R., Aunis, D., Zwiller, J., 2004. Cocaine downregulates the expression of the mitochondrial genome in rat brain. *Ann. N. Y. Acad. Sci.* 1025, 345–350.
- Dietrich, J.B., Mangeol, A., Revel, M.O., Burgun, C., Aunis, D., Zwiller, J., 2005. Acute or repeated cocaine administration generates reactive oxygen species and induces antioxidant enzyme activity in dopaminergic rat brain structures. *Neuropharmacology* 48, 965–974.
- Eisch, A.J., Marshall, J.F., 1998. Methamphetamine neurotoxicity: dissociation of striatal dopamine terminal damage from parietal cortical cell body injury. *Synapse* 30, 433–445.
- Enoksson, M., Robertson, J.D., Gogvadze, V., Bu, P., Kropotov, A., Zhivotovsky, B., Orrenius, S., 2004. Caspase-2 permeabilizes the outer mitochondrial membrane and disrupts the binding of cytochrome c to anionic phospholipids. *J. Biol. Chem.* 279, 49575–49578.
- Fleckenstein, A.E., Gibb, J.W., Hanson, G.R., 2000. Differential effects of stimulants on monoaminergic transporters: pharmacological consequences and implications for neurotoxicity. *Eur. J. Pharmacol.* 406, 1–13.
- He, J., Xiao, Y., Casiano, C.A., Zhang, L., 2000. Role of mitochondrial cytochrome c in cocaine-induced apoptosis in coronary artery endothelial cells. *J. Pharmacol. Exp. Ther.* 295, 896–903.
- Hester, R., Garavan, H., 2004. Executive dysfunction in cocaine addiction: evidence for discordant frontal, cingulate, and cerebellar activity. *J. Neurosci.* 24, 11017–11022.
- Jayanthi, S., Deng, X., Noailles, P.A., Ladenheim, B., Cadet, J.L., 2004. Methamphetamine induces neuronal apoptosis via cross-talks between endoplasmic reticulum and mitochondria-dependent death cascades. *FASEB J.* 18, 238–251.
- Jimenez, A., Jorda, E.G., Verdager, E., Pubill, D., Sureda, F.X., Canudas, A.M., Escubedo, E., Camarasa, J., Camins, A., Pallas, M., 2004. Neurotoxicity of amphetamine derivatives is mediated by caspase pathway activation in rat cerebellar granule cells. *Toxicol. Appl. Pharmacol.* 196, 223–234.
- Kolb, B., Pellis, S., Robinson, T.E., 2004. Plasticity and functions of the orbital frontal cortex. *Brain Cogn.* 55, 104–115.
- Koob, G.F., 1992. Drugs of abuse: anatomy, pharmacology and function of reward pathways. *Trends Pharmacol. Sci.* 13, 177–184.
- Krasnova, I.N., Ladenheim, B., Cadet, J.L., 2005. Amphetamine induces apoptosis of medium spiny striatal projection neurons via the mitochondria-dependent pathway. *FASEB J.* 19, 851–853.
- Kroemer, G., Martin, S.J., 2005. Caspase-independent cell death. *Nat. Med.* 11, 725–730.
- Lehrmann, E., Oyler, J., Vawter, M.P., Hyde, T.M., Kolachana, B., Kleinman, J.E., Huestis, M.A., Becker, K.G., Freed, W.J., 2003. Transcriptional profiling in the human prefrontal cortex: evidence for two activation states associated with cocaine abuse. *Pharmacogenomics* 3, 27–40.
- Li, H., Xu, L., Dunbar, J.C., Dhabuwala, C.B., 2003. Role of mitochondrial cytochrome c in cocaine-induced apoptosis in rat testes. *Urology* 61, 646–650.
- Lyvers, M., 2000. “Loss of control” in alcoholism and drug addiction: a neuroscientific interpretation. *Exp. Clin. Psychopharmacol.* 8, 225–249.
- Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65, 55–63.
- Nassogne, M.C., Evrard, P., Courtroy, P.J., 1998. Selective direct toxicity of cocaine on fetal mouse neurons. Teratogenic implications of neurite and apoptotic neuronal loss. *Ann. N. Y. Acad. Sci.* 846, 51–68.
- Oliveira, M.T., Rego, A.C., Morgadinho, M.T., Macedo, T.R., Oliveira, C.R., 2002. Toxic effects of opioid and stimulant drugs on undifferentiated PC12 cells. *Ann. N. Y. Acad. Sci.* 965, 487–496.
- Oliveira, M.T., Rego, A.C., Macedo, T.R., Oliveira, C.R., 2003. Drugs of abuse induce apoptotic features in PC12 cells. *Ann. N. Y. Acad. Sci.* 1010, 667–670.
- Olsen, G.D., 1995. Potential mechanisms of cocaine-induced developmental neurotoxicity: a minireview. *Neurotoxicology* 16, 159–167.
- Pacifici, R., Fiaschi, A.I., Micheli, L., Centini, F., Giorgi, G., Zuccaro, P., Pichini, S., Di, C.S., Bacosi, A., Cerretani, D., 2003. Immunosuppression and oxidative stress induced by acute and chronic exposure to cocaine in rat. *Int. Immunopharmacol.* 3, 581–592.
- Palmeira, C.M., Moreno, A.J., Madeira, V.M., Wallace, K.B., 1996. Continuous monitoring of mitochondrial membrane potential in hepatocyte cell suspensions. *J. Pharmacol. Toxicol. Methods* 35, 35–43.
- Rolls, E.T., 2004. The functions of the orbitofrontal cortex. *Brain Cogn.* 55, 11–29.

- Sedmak, J.J., Grossberg, S.E., 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal. Biochem.* 79, 544–552.
- Steketee, J.D., 2003. Neurotransmitter systems of the medial prefrontal cortex: potential role in sensitization to psychostimulants. *Brain Res. Brain Res. Rev.* 41, 203–228.
- Stout, J.C., Busemeyer, J.R., Lin, A., Grant, S.J., Bonson, K.R., 2004. Cognitive modeling analysis of decision-making processes in cocaine abusers. *Psychon. Bull. Rev.* 11, 742–747.
- Stumm, G., Schlegel, J., Schafer, T., Wurz, C., Mennel, H.D., Krieg, J.C., Vedder, H., 1999. Amphetamines induce apoptosis and regulation of bcl-x splice variants in neocortical neurons. *FASEB J.* 13, 1065–1072.
- Su, J., Li, J., Li, W., Altura, B.T., Altura, B.M., 2003. Cocaine induces apoptosis in cerebral vascular muscle cells: potential roles in strokes and brain damage. *Eur. J. Pharmacol.* 482, 61–66.
- Sulzer, D., Rayport, S., 1990. Amphetamine and other psychostimulants reduce pH gradients in midbrain dopaminergic neurons and chromaffin granules: a mechanism of action. *Neuron* 5, 797–808.
- Sulzer, D., Sonders, M.S., Poulsen, N.W., Galli, A., 2005. Mechanisms of neurotransmitter release by amphetamines: a review. *Prog. Neurobiol.* 75, 406–433.
- Swerdlow, R.H., Parks, J.K., Cassarino, D.S., Maguire, D.J., Maguire, R.S., Bennett, J.P., Davis, R.E., Parker, W.D., 1997. Cybrids in Alzheimer's disease: a cellular model of the disease? *Neurology* 49, 918–925.
- Tzschentke, T.M., Schmidt, W.J., 2000. Functional relationship among medial prefrontal cortex, nucleus accumbens, and ventral tegmental area in locomotion and reward. *Crit. Rev. Neurobiol.* 14, 131–142.
- Volkow, N.D., Ding, Y.S., Fowler, J.S., Wang, G.J., 1996. Cocaine addiction: hypothesis derived from imaging studies with PET. *J. Addict. Dis.* 15, 55–71.
- Xiao, Y., He, J., Gilbert, R.D., Zhang, L., 2000. Cocaine induces apoptosis in fetal myocardial cells through a mitochondria-dependent pathway. *J. Pharmacol. Exp. Ther.* 292, 8–14.
- Yuan, C., Acosta Jr., D., 2000. Effect of cocaine on mitochondrial electron transport chain evaluated in primary cultures of neonatal rat myocardial cells and in isolated mitochondrial preparations. *Drug Chem. Toxicol.* 23, 339–348.
- Zachor, D.A., Moore, J.F., Brezaussek, C., Theibert, A., Percy, A.K., 2000. Cocaine inhibits NGF-induced PC12 cells differentiation through D(1)-type dopamine receptors. *Brain Res.* 869, 85–97.
- Zaragoza, A., ez-Fernandez, C., Alvarez, A.M., Andres, D., Cascales, M., 2001. Mitochondrial involvement in cocaine-treated rat hepatocytes: effect of N-acetylcysteine and deferoxamine. *Br. J. Pharmacol.* 132, 1063–1070.
- Zhivotovsky, B., Orrenius, S., 2005. Caspase-2 function in response to DNA damage. *Biochem. Biophys. Res. Commun.* 331, 859–867.