MODELING CHRONIC BRAIN EXPOSURE TO AMPHETAMINES USING PRIMARY RAT NEURONAL CORTICAL CULTURES

T. B. NOGUEIRA, a S. DA COSTA ARAÚJO, a F. CARVALHO, a,b F. C. PEREIRA, b E. FERNANDES, c M. L. BASTOS, a V. M. COSTA a AND J. P. CAPELA a,b,c,d

a REQUIMTE (Rede de Química e Tecnologia), Laboratório de Toxicologia, Departamento de Ciências Biológicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
b Farmacologia e Terapêutica Experimental, Instituto Biomédico de Investigação da Luz e Imagem (IBIL), Faculdade de Medicina, Universidade de Coimbra, 3000-548 Coimbra, Portugal
c REQUIMTE (Rede de Química e Tecnologia), Laboratório de Química Aplicada, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua de Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal
d Faculdade de Ciências da Saúde, Universidade Fernando Pessoa, Rua Carlos da Maia, 296, 4200-150 Porto, Portugal

Abstract—Amphetamine-type psychostimulants (ATS) are used worldwide by millions of patients for several psychiatric disorders. Amphetamine (AMPH) and “ecstasy” (3,4-methylenedioxymethamphetamine or MDMA) are common drugs of abuse. The impact of chronic ATS exposure to neurons and brain aging is still undisclosed. Current neuronal culture paradigms are designed to access acute ATS toxicity. We report for the first time a model of chronic exposure to AMPH and MDMA using long-term rat cortical cultures. In two paradigms, ATS were applied to neurons at day 1 in vitro (DIV) (0, 1, 10 and 100 μM of each drug) up to 28 days (200 μM was applied to cultures up to 14 DIV). Our reincubation protocol assured no decrease in the neuronal media’s drug concentration. Chronic exposure of neurons to concentrations equal to or above 100 μM of ATS up to 28 DIV promoted significant mitochondrial dysfunction and elicited neuronal death, which was not prevented by glutamate receptor antagonists at 14 DIV. ATS failed to promote accelerated senescence as no increase in β-galactosidase activity at 21 DIV was found. In younger cultures (4 or 8 DIV), AMPH promoted mitochondrial dysfunction and neuronal death earlier than MDMA. Overall, AMPH proved more toxic and was the only drug that decreased intraneuronal glutathione levels. Meanwhile, caspase 3 activity increased for either drug at 200 μM in younger cultures at 8 DIV, but not at 14 DIV. At 8 DIV, ATS promoted a significant change in the percentage of neurons and astroglia present in culture, promoting a global decrease in the number of both cells. Importantly, concentrations equal to or below 10 μM of either drug did not promote neuronal death or oxidative stress. Our paradigm of neuronal cultures long-term exposure to low micromolar concentrations of ATS closely reproduces the in vivo scenario, being valuable to study the chronic impact of ATS. © 2014 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: amphetamines, “ecstasy”, neurotoxicity, cortical neurons, chronic exposure, long-term neuronal cultures.

INTRODUCTION

Amphetamine-type psychostimulants (ATS) are used worldwide by millions of patients in the clinical treatment of narcolepsy, obesity, and attention deficit hyperactivity disorder. Nonetheless, concerns persist about the high abuse potential and neurotoxicity of these molecules (Fleckenstein et al., 2007; Capela et al., 2009). ATS are the second major class of illicit drugs consumed for recreational purposes in the world according to the United Nations Office on Drugs and Crime (UNODC, 2012). In terms of prevalence, ATS (excluding “ecstasy”) showed an estimated prevalence of 0.3–1.2% (between 14.3 and 52.5 million users) (UNODC, 2012). Global use of 3,4-methylenedioxymethamphetamine (MDMA; “ecstasy”) and related substances was estimated at 0.2–0.6% of the population aged 15–64 (between 10.5 and 28 million users) (UNODC, 2012).

Amphetamine (AMPH) and MDMA cause monoamine release in the nervous system, particularly noradrenaline, dopamine (DA), and serotonin (5-HT) (Fleckenstein et al., 2007; Capela et al., 2009). Chronic AMPH abuse is reported to be associated with attention and memory impairments, as well as deficits in decision-making cognition (Ornstein et al., 2000; Krasnova et al., 2005; Fleckenstein et al., 2007). Reports have shown long-term impairments in memory and learning in human MDMA abusers, in particular, working memory, planning ability, and executive control, which persist long after cessation of drug use (Reneman et al., 2000; Capela et al., 2009).

5-HT, serotonin; Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp-p-nitroanilide; AMPH, amphetamine; ANOVA, analysis of variance; ATS, amphetamine-type psychostimulants; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DA, dopamine; DIV, day in vitro; EDTA, ethylenediaminetetraacetic acid; GFAP, glial fibrillary acidic protein; GSH, total glutathione; GSSG, oxidized glutathione; HEPES, 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; MAP2, microtubule-associated protein 2; MDMA, 3,4-methylenedioxymethamphetamine (“ecstasy”); MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline.
These chronic neurocognitive deficits seem to be attributable to the well-described neurotoxic effects of both drugs in animal models. Particularly, acute and sub-acute administration of AMPH and MDMA to rodents can cause long-term DA and 5-HT depletion, which is accompanied by decreases in tyrosine hydroxylase and tryptophan hydroxylase immunoreactivity, loss of DA and 5-HT transporters, and degeneration of striatal dopaminergic and serotonergic terminals (Ryan et al., 1990; Pereira et al., 2006; Wan et al., 2006; Alves et al., 2007; Fleckenstein et al., 2007). Additionally, the degeneration of cell bodies, including pyramidal cells, in the cortex of AMPH and MDMA-treated animals has been documented in acute as well as in sub-acute settings (Ryan et al., 1990; Schmued, 2003; Belcher et al., 2005; Krasnova et al., 2005; Warren et al., 2007). We have demonstrated that MDMA acutely triggered apoptosis in cultured cortical neurons (Capela et al., 2006a,b, 2007a). Moreover, metabolism is known to play an important role in both MDMA and AMPH effects, and may play an important role in their neurotoxicity (Law and Moody, 2000; Capela et al., 2007b).

The outcomes from chronic use of ATS are unknown or uncertain, and they are generally not evaluated by the current laboratory cell culture paradigms. In particular, neuronal cell culture studies are generally designed to evaluate the acute effects of drugs by exposing neurons to high levels of ATS (Stumm et al., 1999; Jimenez et al., 2004; Capela et al., 2006b, 2013; Wang et al., 2008; Ruscher et al., 2011). These studies while important, fail to mimic the chronic exposure to ATS that occurs after long-term use of these drugs. Herein, we report for the first time the development of an in vitro model using rat cortical cultured neurons, up to 28 days of culture, to examine the neurotoxic effects of chronic exposure to low concentrations of MDMA or AMPH.

**EXPERIMENTAL PROCEDURES**

**Materials**

Materials for cell cultures were obtained from the following sources: neurobasal medium and supplement B27 from Gibco (Invitrogen, Paisley, UK); modified Eagle’s medium, phosphate-buffered saline (PBS), HEPES buffer, trypsin/EDTA, penicillin/streptomycin, L-glutamine, collagen-G and poly-L-lysine from Biochrom (Berlin, Germany); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 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), reduced and oxidized glutathione (GSSG) and Senescent Cells Staining Kit (CS0030 Sigma–Aldrich) from Sigma–Aldrich (St Louis, MO, USA). The AMPH used was d-AMPH sulfate and was obtained from Merck AG (Darmstadt, Germany). MDMA (HCl salt) was purified from high purity MDMA tablets (kindly provided by the Portuguese Criminal Police Department) by organic solvent extraction procedures and converted into the HCl salt. The obtained salt was pure and fully characterized by nuclear magnetic resonance and mass spectrometry methodologies, as we previously reported (Capela et al., 2007b). Six- and 48-multwell plates, as well as sterile pipettes and filters were obtained from Corning Costar (Corning, NY, USA). Reagents for the immunocytochemistry were purchased from the following sources: normal goat serum (G9023), polyclonal anti-glial fibrillary acidic protein (GFAP) antibody produced in rabbit (G9269) and the monoclonal anti-microtubule-associated protein 2 (MAP2) antibody produced in mouse (M4403) were obtained from Sigma–Aldrich; Alexa Fluor 488 goat anti-mouse IgG (A-11001) and Alexa Fluor 546 goat anti-rabbit IgG (A-11035) were obtained from Life Technologies Europe B.V. (Netherlands). All other chemicals were purchased from Sigma–Aldrich from the highest grade commercially available (St Louis, MO, USA).

**Cell culture**

All experiments were carried out in compliance with current European directives on animal experimentation (2010/63/EU). Primary neuronal cultures of the cortex were prepared from embryos (E-17/E-19) of Wistar rats. Cultures were prepared using previously published methods (Capela et al., 2006a,b). Briefly, meninges were removed, the cerebral cortex was dissected, and the tissue obtained was incubated for 15 min in trypsin/EDTA (0.05/0.02% w/v in PBS) at 37°C; the cortex was 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 (210g for 2 min), resuspended in a density of 1.5 × 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 with cultivating medium (starter medium without glutamate).

**Experimental protocol paradigm 1 – exposure to drugs up to 14 days in vitro**

One day after seeding, taken as the 1st day in vitro (DIV), neurons were exposed to AMPH or MDMA salts (dissolved in sterilized deionised water) with the following final concentrations: 1, 10, 100, and 200 µM. In order to evaluate the neuronal effects of continuous exposure to drugs, cultures were also fed at the 4 and 8 DIV by addition of fresh medium that, in treated cells, contained the appropriate concentration of the drug. This protocol of reincubation with drugs at each fourth day assured the drug concentration in the media. Four, 8, and 14 DIV were considered time-points for the
assessments. The diagram of the protocol described for paradigm 1 is depicted in Fig. 1A.

The concentrations of AMPH and MDMA were selected in accordance with those found in the brain of laboratory animals following ATS administration. Clausing et al. (1995) measured the levels of D-AMPH in the substantia nigra and caudate/putamen of rats 50 min after subcutaneous (s.c.) administration, at different environmental temperatures using microdialysis. At the environmental temperature of 23°C, one or three doses (every 2 h) of 5-mg/kg produced peak tissue levels of 36 ± 6 μM and 73 ± 10 μM in the substantia nigra and 25 ± 4 and 50 ± 8 μM in caudate/putamen, respectively. Importantly, at the environmental temperature of 10°C, three doses of 5 mg/kg resulted in levels that were three to five times higher than at 23°C in the caudate/putamen and substantia nigra (Clausing et al., 1995), being 200 μM attainable in vivo. In rats, the 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). Following a rational approach of allomorphic scaling used by Ricaurte et al. (2000), a dose of 20 mg/kg of MDMA in a rat is equivalent to a dose of 98 mg in a 75-kg human. The latest European Union report on MDMA mentioned that the seized pills during 2012 had a mean range of 57–102 mg, thus 20 mg/kg in a rat can be equivalent to a single pill in a human (EMCDDA, 2014). Unlike AMPH general use by Humans, MDMA is generally consumed in binges during weekends, but there are reports of drug abusers using the drug on a continuous basis for more than 48 h (Parrott, 2006). Moreover, considering that the half-life of MDMA racemate is about 12 h (de la Torre et al., 2004) and disproportionate higher levels of MDMA are found in the brain versus the plasma (Chu et al., 1996), one can assume that use of MDMA several times a week can lead to continuous brain exposure to the drug. Therefore, the concentrations of AMPH and MDMA used in our protocol of long-term exposure match those attained in vivo, providing a rational for our neuronal culture model to mimic the chronic use of either drug.

For experiments with the glutamate antagonists, neurons were exposed at 1 DIV to 200 μM AMPH or MDMA until the 14th DIV, time-point at which both MTT and LDH assays were conducted. The antagonists MK-801 (1 μM), a noncompetitive antagonist of the N-methyl-D-aspartate (NMDA) receptor, and/or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM), a competitive AMPA/kainate receptor antagonist, were only added to the cultures at 4 DIV. Neurons were only exposed to the antagonists at 4 DIV given the importance of glutamate for neuronal growth and

![Diagram of exposure of cortical neuronal cultures to drugs. To assure the maintenance of the drug concentration in the neuronal medium in both paradigms, cultures were feed, at the indicated times, with fresh medium containing the appropriate concentration of the drug. (A) Paradigm of exposure 1, where neurons were exposed continuously after the first day in vitro (DIV) until 4, 8 and 14 DIV to AMPH or MDMA (1, 10, 100, and 200 μM). (B) Paradigm of exposure 2, where neurons were exposed continuously after the first day in vitro (DIV) until 21 or 28 DIV to AMPH or MDMA (1, 10 or 100 μM). In the case of exposures until 21 DIV cultures had their last fed at 16 DIV.](https://example.com/diagram1.png)
development in the first days of cultures (Brewer, 1995). At 8 DIV, fresh medium containing both the antagonists and the drugs was added to treated cells until 14 DIV. The concentrations of the antagonists were chosen in accordance with their receptor selectivity and previous papers conducted with neuronal cultures (Capela et al., 2006b; Marino et al., 2007).

Measurement of MDMA and AMPH concentrations in the neuronal medium by high-performance liquid chromatography linked with UV detection (HPLC–UV)

In order to guarantee that the concentrations of drugs remained constant during the several days that cultures had to remain in the incubator for the experiments, the highest concentrations (100 and 200 μM) of AMPH and MDMA in the neuronal medium were quantified by HPLC at the time-points of exposure, 8 and 14 DIV. The quantification of AMPH and MDMA in culture medium was performed through a previously validated method, with minor modifications (Bogusz et al., 1997). To 750 μL of medium was added 250 μL of perchloric acid 10%. The protein pellet obtained after 10-min centrifugation at 13,000 rpm was discarded, and the perchloric acid supernatant was stored at −80 °C until analysis. HPLC analysis was performed using a Waters 2690 separation module (Waters, Milford, MA, USA), and a commercially-prepacked reverse phase cartridge of 250 mm × 4.6 mm, Waters Spherisorb RP-18 (5 μm) ODS2 column. Isocratic elution was performed at a flow rate of 1.0 ml/min, at room temperature. For the quantification of AMPH and MDMA, a photodiode array detector (Waters model 996) was used. The mobile phase was 50 mM ammonium formate buffer (pH 3.0) plus acetonitrile (Bogusz et al., 1997) (8% acetonitrile for culture medium of MDMA-incubated neurons and 10% for culture medium of AMPH-incubated neurons). The wavelengths used in the UV detector were 210 and 279 nm for AMPH and MDMA, respectively. These wavelengths allowed better quantification of pure AMPH and/or MDMA standards spiked in the neuronal medium of control cells. Each sample in 2.5% perchloric acid was injected into the HPLC system by an auto sampler. The AMPH and MDMA concentrations in neuronal medium were assessed from calibration curves constructed with at least six concentrations of each drug and performed in the same day that samples were to be injected.

The analysis of MDMA metabolites was made according to Barbosa et al. (2013) using the previously mentioned HPLC system coupled to coulometric analysis. For the coulometric analysis of the metabolites, a Colouchem II (ESA, Chelmsford, USA) equipped with a guard cell (ESA 5020) and analytical cell (ESA 5011A) electrochemical detector were used. The electrochemical potential settings of the Colouchem II detector were the following: guard cell, +0.5 V; detector 1, −0.075 V and detector 2 (analytical detector), +0.45 V, as previously referred. The mobile phase for the analysis of the metabolites was 10% methanol in 50 mM citric acid, 0.46 mM octanesulfonic acid, adjusted to pH 3.0.

Experimental protocol paradigm 2 – exposure to drugs up to 28 DIV

In order to evaluate the neuronal effects of long-term exposure to drugs, we developed a second paradigm using long-term neuronal cultures maintained up to 28 DIV. In accordance with the previous first set of experiments, at 1 DIV, neurons were exposed to AMPH or MDMA (dissolved in sterilized deionised water) with the following final concentrations: 1, 10 and 100 μM. Cultures were fed with fresh medium at every 4th day at 4, 8, 12, 16, 20 and 24 DIV in the case of exposures up to 28 DIV. Cultures maintained until 21 DIV had their last medium change at 16 DIV. At every feeding, fresh medium in the treated cells contained the appropriate concentration of the drug in order to guarantee the drug concentration throughout the protocol. At 21 DIV and 28 DIV end-points, several biochemical and histological determinations were performed. The diagram of the protocol described for paradigm 2 is depicted in Fig. 1B.

Immunocytochemistry

For immunocytochemistry assays, cultures were grown in 48-well plates and used at the selected time-points of 8 and 21 DIV. Before fixation, the medium was removed, cells were washed with PBS, and then fixed by adding ice-cold methanol for 15 min at −20 °C. Cells were then permeabilized with 0.1% Triton X-100 in PBS for ten minutes, and washed with PBS. To saturate unspecific binding sites, cells were placed in a shaker for 1 h at room temperature with a blocking solution containing 10% normal goat serum in PBS. This was followed by an overnight incubation at −4 °C with both primary antibodies at the same time, anti-GFAP (1:200) and anti-MAP2 (1:200). After five washes with PBS for five minutes, cells were incubated in a shaker for 1 h at room temperature with both secondary antibodies, Alexa Fluor 546 goat anti-rabbit IgG (1:500) and Alexa Fluor 488 goat anti-mouse IgG (1:500) antibodies. Both primary and secondary antibodies were diluted in 3% normal goat serum in PBS. After several washes with PBS, the staining was analyzed and microphotographs were taken with a fluorescence inverted microscope (Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera, Nikon, Japan). Images of neurons and astrocytes in culture were taken in the same field using a 200× magnification. The quantification of the ratio of MAP2-positive cells, taken as neurons, and GFAP-positive cells, taken as astroglial cells, was made by counting at least four fields per well in at least two wells per condition (at least 1000 neurons were counted per condition). Images were processed with NIS-Elements Microscope Imaging Software (Nikon, Tokyo, Japan).

Cellular viability assays – LDH and MTT assays

Cell cultures were assessed morphologically by phase contrast microscopy at every feeding day. Cell damage was assessed quantitatively by the measurement of LDH release into the medium (as a measure of cell membrane integrity) using a kinetic measurement assay,
as described previously (Capela et al., 2006b). The ratio of cell death expressed in percentage was calculated by dividing the LDH leakage into the medium through the respective total cellular LDH, whose value was set to 100% of cell death. Total LDH activity was obtained following the full kill with Triton X-100 0.5% for 30 min per well (Ferreira et al., 2013). Also, mitochondrial dysfunction was assessed by the measurement of MTT metabolization assay, as described previously (Capela et al., 2006a). Data obtained are presented as percentage of control vehicle-treated cultures, which were set to 100%.

Though the LDH and MTT assays are widely used in cell culture, they have several limitations (Galluzzi et al., 2005; Costa et al., 2009b). Therefore, we conducted several assays as to circumvent the limitation of each test. LDH assay results were expressed as the ratio of extracellular LDH versus total LDH after the full kill. Additionally, as a complementary measurement of cell death, the evaluation of caspase 3 activity was also performed. Though the MTT reduction assay is generally regarded as a measure of cell death/proliferation, given the importance of the mitochondrial chain, in particular complex I and II to the metabolism of MTT, the formation of the formazan has been regarded as a measure of mitochondrial dysfunction (Berridge et al., 2005; Costa et al., 2009b).

Caspase 3 assay
Caspase 3 assay was based on a previously described colorimetric assay (Capela et al., 2007b). The hydrolysis of the peptide substrate Ac-DEVD-pNA by caspase 3 results in the release of the p-nitroaniline (pNA) moiety, which has an absorbance at 405 nm. Per condition approximately $5 \times 10^5$ cells were collected. Protein concentration in the lysates was determined using the Bio-Rad kit (“DC Protein Assay”), and using bovine serum albumin solutions as standards. Caspase activity is presented as optical density per quantity of protein contained in the cellular lysates.

Total and GSSG measurement
Measurement of intracellular total glutathione (GSHT) levels was carried out according to a previously published method (Capela et al., 2007b). Briefly, approximately $2.5 \times 10^5$ cells were scraped per condition and pelleted cells were stored in perchloric acid (final concentration 5%). Both GSHT and oxidized GSSG levels were measured. For GSSG, the acidic supernatant was pre-treated with 5% 2-vinylpyridine during 1 h at 4 °C, to block free SH groups. Glutathione content was measured by the rate of colorimetric formation of 5′-thio-2-nitrobenzoic acid (TNB) at 415 nm using a microplate reader. GSHT levels and GSSG contents were normalized to the total protein content.

Senescence cell staining based on histochemical stain of β-galactosidase activity
Identification of senescent cells was performed with Senescent Cells Staining Kit, in accordance with the manufacturer’s instructions, by histochemical detection of β-galactosidase at pH 6. β-Galactosidase activity detected by histochemical staining is a measure of increased residual lysosomal activity and has been used to identify senescent cells in culture (Chernova et al., 2006). Briefly, cells were cultured in 48-well plates and fixed with the fixative provided by the kit during 10 min at room temperature. Following the washing steps, the staining solution containing X-GAL was added to each well, the plate sealed with parafilm, and incubated overnight at 37 °C in a CO₂ free incubator.

Phase-contrast microphotographs were taken following the staining procedure (Nikon Eclipse TS100 equipped with a Nikon DS-Fi1 camera). In every experiment, each condition had three wells, from each well three photos were taken and blue-stained neurons were counted as senescent cells. Results were expressed as the percentage of X-GAL-positive neurons in relation to the whole number of cells present in the same photo bearing a neuronal morphology.

Statistical analysis
Results are presented as mean ± SD. Data were obtained from three to five independent experiments to pool data from several neuronal cultures obtained and seeded in different days. Due to the nature of the preparation of primary cortical neurons obtained from rat embryos and the number of cells achieved, data at each time-point were conducted with different cultures obtained at different periods. A two-way analysis of variance (ANOVA) was used to compare means from different treatment groups in graphics containing two variables, followed by the Bonferroni post hoc test, if a significant p value had been obtained. In particular, for the data of glutamate antagonists and the relative percentage of astroglial cells, the statistical analysis performed was the one-way ANOVA, followed by Bonferroni post hoc test. In all other data, containing one variable (treatment), and since data not always passed normality tests, the Kruskal–Wallis test (ANOVA on ranks) was performed, followed by Dunn’s post hoc test. In each figure legend are mentioned the details of the F values for each variable in the ANOVA tests, and the Kruskal–Wallis statistic value for the nonparametric ANOVA. Tests were performed using GraphPad Prism 6.0 software (CA, USA). Statistical significance was accepted at p values less than 0.05.

RESULTS
AMPH and MDMA concentrations did not decrease in the neuronal medium over time with our exposure protocol
We measured the concentration of AMPH and MDMA in the neuronal medium at two selected time-points, 8 and 14 DIV (Fig. 2). These correspond to the latter time-points of cells’ exposure to the drugs in paradigm 1. To assure an accurate and measurable assessment of the drug concentration in the medium, the neuronal cultures exposed to 100 and 200 μM were selected. As it can be seen in the Fig. 2C, D, the method could easily
discriminate and assure reliable measures of the peaks corresponding to AMPH and MDMA in the chromatograms. The retention times of pure standards of AMPH and MDMA were 16.5 and 37 min, respectively, in our chromatographic conditions. In both AMPH (Fig. 2A) and MDMA (Fig. 2B), the concentrations measured in the neuronal medium at 8 and 14 DIV were the expected for 100 µM. For the cells exposed to 200 µM of both drugs, while at 8 DIV the concentrations remained at the expected levels, at 14 DIV there was a slight, but statistically significant increase in the drug concentration in the medium from the 8 to the 14 DIV. At 14 DIV for both drugs the concentration in the medium was about 15% higher than the expected 200 µM.

Additionally, measurements were performed by HPLC coupled to electrochemical detection in the collected cell culture medium to check whether the catechol metabolites of MDMA, namely N-methyl-α-methylidopamine and α-methylidopamine, could be detected. For the tested experimental conditions, the formation of MDMA metabolites in these cultures was below the limit of detection of the method (data not shown). The method used in our laboratory can detect concentrations lower than 1 µM (Barbosa et al., 2013). Therefore, we can assume that these metabolites are not formed in measurable amounts in cortical neuronal cultures.

The percentage of glia in the neuronal culture approximately doubled from 8 to 21 DIV. ATS promoted changes in the relative number of neurons and astroglial cells at 8 DIV.

We have estimated the relative abundance of astroglial cells in our cultures at two time-points: 8 and 21 days in culture (Fig. 3A). Following MAPII and GFAP labeling of cultured cells, we found at 8 DIV that around 18% of the whole cellular population were GFAP positive (Table 1),
meanwhile at 21 DIV the percentage of astroglial cells increased to 30%. Older cultures have a higher percentage of GFAP-positive cells, and glia present a denser network at that later stage. However, this increase in the percentage of GFAP-positive cells did not correlate to an increase in the number of astroglial cells per mm$^2$; in reality there was a modest decrease in the total number of GFAP-positive cells from 8 to 21 DIV (Fig. 3B). Importantly, the number of neurons presented at 21 DIV is roughly half of those at 8 DIV (Fig. 3B).

The relative number of GFAP- and MAP II-positive cells was evaluated following exposure of cultures to either 100 μM of AMPH or MDMA at 8 DIV (Table 1). We found a significant difference in terms of the ratio of

<table>
<thead>
<tr>
<th>8 DIV</th>
<th>Control (%)</th>
<th>100 μM AMPH (%)</th>
<th>100 μM MDMA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAPII</td>
<td>82 ± 2</td>
<td>86 ± 3$^*$</td>
<td>87 ± 3$^*$</td>
</tr>
<tr>
<td>GFAP</td>
<td>18 ± 2</td>
<td>14 ± 3$^*$</td>
<td>13 ± 3$^*$</td>
</tr>
</tbody>
</table>

Changes in the ratio of neurons and astroglial cells promoted by AMPH or MDMA at 8 DIV. Cultures of cortical neurons were exposed continuously after the first day until 8 DIV to AMPH or MDMA (100 μM). Results were obtained after counting more than a 1000 neurons in eight different fields. Statistical analysis was conducted by a one-way ANOVA ($F_{(2,21)} = 5.38, p < 0.05$ for MAPII, and $F_{(2,21)} = 5.38, p < 0.05$ for GFAP), followed by Bonferroni post hoc test. $p < 0.05$ treatment vs. control.

Fig. 3. The relative abundance of astroglial cells in the cultures at two time-points: 8 and 21 days in culture. (A) Following MAPII, neuronal specific marker, and GFAP, astroglial marker, immunocytochemistry microphotographs were taken at 8 DIV (left panel) and 21 DIV (right panel) (Scale bar = 100 μm). (B) Number of neurons and astroglial cells present in culture per mm$^2$ at 8 and 21 DIV.
MAPII-positive versus GFAP-positive cells to controls following exposure to drugs. There was a significant decrease in the percentage of astroglial cells in comparison to control cultures at 8 DIV (Table 1). In accordance, either drug promoted a similar decrease of ~15% and ~40% in the number of neurons and astroglial cells, respectively, in comparison to 8 DIV controls. Globally after exposure to either drug, there was a decrease of about 20% in total number of cells present in culture at 8 DIV.

Fig. 4. AMPH- and MDMA-induced mitochondrial dysfunction and cell death to cortical neurons. Cultures of cortical neurons were exposed continuously after the first day until 4, 8 and 14 DIV to AMPH or MDMA (1, 10, 100, and 200 µM). (A, B) Mitochondrial dysfunction evaluated by the MTT assay promoted by AMPH or MDMA, respectively. (C, D) Percentage of cell death evaluated by the LDH assay after AMPH or MDMA exposure, respectively. Results were obtained from three to four different and independent experiments, each experiment having six different culture wells per condition. A two-way ANOVA for AMPH (A) found a significant effect of time (F(2,255) = 30.0, p < 0.0001) and of concentration (F(4,255) = 203.9, p < 0.0001), and a significant interaction (p < 0.0001), and for (C) found a significant effect of time (F(2,345) = 468.1, p < 0.0001), and of concentration (F(4,345) = 22.49, p < 0.0001), and no interaction (p > 0.05), and for (E) found a significant effect of time (F(2,345) = 275.6, p < 0.0001), and of concentration (F(4,345) = 6.06, p < 0.0001), and no interaction (p > 0.05); as for MDMA (B) there was a significant effect of time (F(2,345) = 13.5, p < 0.0001) and of concentration (F(4,345) = 101.5, p < 0.0001), and a significant interaction (p < 0.0001), and for (D) a significant effect of time (F(2,345) = 19.2, p < 0.0001), and of concentration (F(4,345) = 6.06, p < 0.0001), and no interaction (p > 0.05) (p < 0.05), and for (F) a significant effect of time (F(2,315) = 314.1, p < 0.0001) and of concentration (F(4,315) = 10.6, p < 0.0001), and a significant interaction (p < 0.0001). A two-way ANOVA was followed by a Bonferroni post hoc test (**p < 0.01, +++p < 0.001 concentration vs. control; **p < 0.05, ##p < 0.01, ###p < 0.001 concentration vs. 1 µM; **p < 0.05, ##p < 0.01, ###p < 0.001 concentration vs. 10 µM; +p < 0.05, +p < 0.01, ++p < 0.001 concentration vs. 100 µM).
AMPH promoted mitochondrial dysfunction and cell death at earlier time-points when compared with MDMA in cultures exposed up to 14 DIV

Neurons were exposed to AMPH and MDMA at 1 DIV (concentrations 1, 10, 100 and 200 μM) according to the first protocol of exposure described (paradigm 1, Fig. 1A). At time-points 4, 8 and 14 DIV, mitochondrial dysfunction and cell death were assessed by the MTT and the LDH release assays, respectively (Fig. 4).

In the MTT assay a statistically significant mitochondrial dysfunction induced by AMPH for the concentrations 100 and 200 μM at all time-points tested was seen, being more pronounced at 8 DIV (Fig. 4A). Mitochondrial dysfunction was triggered by the lowest AMPH concentrations (1 and 10 μM) at 8 DIV, but that was a transient effect since at 14 DIV the cells seem to recover the MTT metabolism ability, as no difference could be seen when compared to control cells (Fig. 4A). In the case of MDMA, only for the concentrations of 100 and 200 μM was possible to verify mitochondrial dysfunction, and at 4 and 14 DIV only the 200 μM promoted a significant decrease in MTT metabolization (Fig. 4B). From 8 to 14 DIV for 100 μM of MDMA the cells seem to elicit a different response, and MTT metabolization significantly increased (Fig. 4B). Notably, from the 4 to 8 DIV both drugs induced a time-dependent decrease in MTT metabolism. Importantly, AMPH seems to cause a higher degree of mitochondrial dysfunction than MDMA when looking at the same concentrations and time-points (Fig. 4A, B).

The LDH release assay showed that 100 and 200 μM concentrations of AMPH induced significant neuronal death as soon as 4 DIV (Fig. 4C). Also, at 8 DIV, both 100 and 200 μM of AMPH promoted significant cell death, but at 14 DIV only the higher concentration of AMPH promoted neuronal death. MDMA promoted significant neuronal death at 8 DIV, which was seen for either 100 or 200 μM (Fig. 4D). At the latter time-point, 14 DIV, there was only neuronal death for the highest concentration tested of MDMA. Overall, there was a concentration-dependent neuronal death induced both by AMPH and MDMA at 8 DIV (Fig. 4C, D). Importantly, shorter times of exposure to AMPH were needed to trigger neuronal death than those for MDMA, whereas for longer time-points (8 and 14 DIV) the neuronal death seems to be similar.

To confirm whether ATS could interfere with cellular growth and development of neuronal cultures over the time of exposure, we measured the total LDH activity obtained after the full kill. This measures the LDH of the whole cultured population, including neurons and glia cells (Fig. 4E, F). We verified that only at the time-point 14 DIV, we could measure a significant decrease in total activity of LDH after the full kill for either MDMA or AMPH for the 200 μM concentration (also 100 μM in the case of MDMA) (Fig. 4E, F). Of note the substantial increase in the total LDH activity in control wells with the time of culture, from the 4th to the 14th DIV, as neuronal cultures grow and develop in vitro. Importantly, in control cultures the percentage of cell death decreases substantially from 4 to 8 DIV given the fact that there is an increase in total LDH, thereby affecting the ratio extracellular LDH/total LDH.

Only the highest concentration of AMPH and MDMA promoted an increase in caspase 3 activity in cortical neurons at 8 DIV

Caspase 3 activity was measured to assess whether neuronal death was accompanied by pro-apoptotic signaling. Caspase 3 activity was significantly increased in cultured cortical neurons for 200 μM of AMPH and MDMA at the 8-DIV time-point (Fig. 5A, B). For all other concentrations and time-points, caspase 3 activity was similar to control levels. Interestingly, 10 μM of AMPH could significantly reduce caspase 3 activity at 14 DIV, when compared to control cultures.

When analyzing the activity of caspase 3 in control cells, one can easily see that at 14 DIV the activity is at least twofold higher than at 4 or at 8 DIV, reflecting the ongoing neurodegeneration of cortical neurons under culture conditions.

The highest concentration of AMPH decreased GSHt levels of neuronal cortical cultures in exposures up to 14 DIV

As both ATS are known to elicit oxidative stress, we tested whether AMPH or MDMA could promote changes in the intracellular levels of GSH and in the levels of GSSG. GSH levels were unchanged for all time-points and concentrations equal to or bellow a 100 μM of either drug (Fig. 6A, B). Only the highest concentration of AMPH at both 8 and 14 DIV caused a significant decrease of intracellular GSHt levels (Fig. 6A). No significant changes were seen in the case of MDMA for the same concentration, though there was a tendency for lower intracellular GSHt levels at 8 DIV for 200 μM, it did not reach statistical significance (Fig. 6B).

GSSG levels were not detected at 4 DIV with our method and therefore only data for 8 and 14 DIV are presented (Fig. 6C, D). For both AMPH (Fig. 6C) and MDMA (Fig. 6D), there were no changes in GSSG levels in comparison to controls at all concentrations and time-points, except for 100 μM of AMPH at 8 DIV. At this time-point 100 μM of AMPH promoted a significant decrease in the intracellular levels of GSSG, which was no longer seen at 14 DIV.

Regarding the GSHt levels in control cultured neurons and its evolution over time in culture, GSHt levels at 8 and 14 DIV were approximately twofold higher than at 4 DIV, reflecting the culture development. In contrast, GSSG levels at 8 DIV were approximately twofold higher than at 14 DIV.

Lack of protection by glutamate antagonists against the cell death and mitochondrial dysfunction promoted by AMPH and MDMA

To test whether AMPH and MDMA-induced neurotoxicity involved glutamate excitotoxicity, we incubated cultured cortical neurons with two types of glutamate receptor antagonists: MK-801 (1 μM), a noncompetitive antagonist
Fig. 5. Caspase 3 activity in cortical neuronal cultures exposed to AMPH or MDMA. Cultures of cortical neurons were exposed continuously after the first day until 4, 8 and 14 DIV to AMPH or MDMA (1, 10, 100, and 200 µM), and caspase 3 activity was measured after AMPH, A or MDMA, B exposure. Results were obtained from four different and independent experiments, each experiment having three different culture wells per condition. A two-way ANOVA for AMPH (A) found a significant effect of time ($F_{(2,165)} = 129.4, p < 0.0001$) and of concentration ($F_{(4,165)} = 6.13, p < 0.01$), and a significant interaction ($p < 0.05$); as for MDMA (B) there was a significant effect of time ($F_{(2,165)} = 600.0, p < 0.0001$) and of concentration ($F_{(4,165)} = 34.96, p < 0.0001$), and a significant interaction ($p < 0.0001$). A two-way ANOVA was followed by a Bonferroni post hoc test ($\frac{1}{6} p < 0.05, \frac{1}{3} p < 0.01, \frac{1}{2} p < 0.001$ concentration vs. control; $p < 0.05, \frac{3}{4} p < 0.001$ concentration vs. 1 µM; $\frac{5}{6} p < 0.05$ concentration vs. 10 µM).

Fig. 6. Total glutathione (GSHt) and oxidized glutathione (GSSG) levels in neuronal cultures exposed to AMPH or MDMA. Cultures of cortical neurons were exposed continuously from the first day until 4, 8 and 14 DIV to AMPH or MDMA (1, 10, 100, and 200 µM) and the levels of intracellular GSHt and GSSG were measured. (A, B) GSHt levels after exposure to AMPH or MDMA, respectively. (C, D) GSSG levels after exposure to AMPH or MDMA, respectively. At 4 DIV, the levels of GSSG in neurons were below the limit of quantification of the method. Results were obtained from three to five different and independent experiments, each experiment having two or three different culture wells per condition. A two-way ANOVA for AMPH (A) found a significant effect of time ($F_{(2,165)} = 144.6, p < 0.0001$) and of concentration ($F_{(4,165)} = 11.93, p < 0.0001$), and no interaction ($p > 0.05$), and for (C) a non significant effect of time ($F_{(1,105)} = 1.52, p > 0.05$) and a significant effect of concentration ($F_{(4,105)} = 2.5, p < 0.05$), and a significant interaction ($p < 0.05$); as for MDMA (B) there was a significant effect of time ($F_{(2,165)} = 15.97, p < 0.0001$) but not of concentration ($F_{(4,165)} = 1.57, p > 0.05$), and no interaction ($p > 0.05$), and for (D) a significant effect of time ($F_{(1,110)} = 78.43, p < 0.0001$) but not of concentration ($F_{(4,110)} = 1.56, p > 0.05$), and no interaction ($p < 0.05$). A two-way ANOVA was followed by a Bonferroni post hoc test ($p < 0.05, \frac{1}{3} p < 0.01, \frac{2}{3} p < 0.001$ concentration vs. control; $\frac{3}{4} p < 0.001$ concentration vs. 1 µM; $\frac{5}{6} p < 0.05$ concentration vs. 10 µM; $\frac{7}{6} p < 0.001$ concentration vs. 100 µM).
of the NMDA receptor, and/or CNQX (10 μM), a competitive AMPA/kainate receptor antagonist (Table 2).

Given the fact that the highest neurotoxicity was seen with the concentration of 200 μM, as verified by the MTT and LDH assays, as well as by caspase 3 activity, we selected this concentration and the 14 DIV time-point to assess the putative protection of the antagonists. MK-801 or CNQX alone or even together could not provide any protection against the mitochondrial dysfunction promoted by 200 μM of AMPH or MDMA (Table 2). Also, the antagonists alone or applied together to the neuronal cultures could not prevent the neuronal death induced by 200 μM of AMPH or MDMA, as measured by the LDH release assay (Table 2). Interestingly, CNQX alone was able to decrease neuronal death when compared to control cells.

**Chronic exposure to AMPH and MDMA up to 28 DIV promoted changes in cellular viability only for the highest concentration tested of 100 μM**

In a new set of experiments, neuronal cultures were exposed to AMPH or MDMA at 1 DIV (concentrations 1, 10 and 100 μM) up to 21 and 28 DIV (paradigm 2, Fig. 1B). Since at 8 and 14 DIV, there were already signs of substantial neuronal death following the exposure of cultures to 200 μM, therefore that highest concentration was abandoned in long-term exposure until 21 and 28 DIV.

At the 21 DIV time-point, the MTT assay showed a statistically significant mitochondrial dysfunction induced by 100 μM of AMPH (Fig. 7A), whereas for MDMA exposure the concentration of 100 μM promoted a significant increase in MTT metabolism (Fig. 7B). Interestingly, the percentage of cell death was lower for 100 μM of either drug at this time-point, while lower concentrations did not elicit any change in cellular viability (Fig. 7C, D). This reflects the lower content of extracellular LDH in comparison to controls, as we found at this time-point that the total LDH values, obtained after the full kill of cultures exposed to drugs, were similar to control cultures (data not shown).

We also measured the intracellular GSHt levels in neuronal cultures at 21 DIV. Only the highest concentration of AMPH, 100 μM, promoted a decrease in the GSHt levels (Fig. 7E). On the other hand, MDMA did not alter the intracellular levels of GSHt (Fig. 7F). When comparing the intracellular GSHt levels of control cultures at 21 DIV to the ones found at 8 or 14 DIV, one can easily verify that at 21 DIV GSHt levels are about 2–3 times lower, reflecting the aging of neuronal cultures. As no increase in GSSG levels at the earlier time-points of exposure to drugs was found we did not conduct an analysis of intracellular GSSG levels at 21 DIV.

At 28 DIV, the MTT assay showed a statistically significant mitochondrial dysfunction induced both by AMPH and MDMA for the concentration of 100 μM (Fig. 8A, B). Meanwhile, no changes in terms of percentage of cell death could be seen in the LDH release assay (Fig. 8C, D). In fact, the reduction of cell death verified in the LDH assay at 21 DIV was no longer seen at 28 DIV. Likewise, the MTT assay showed a consistent reduction in the MTT metabolism for 100 μM of both drugs, in contrast to that verified at 21 DIV for MDMA.

**No increase in X-GAL staining of neurons after chronic exposure to AMPH and MDMA until 21 DIV**

As a measure of cellular senescence associated with aging, we counted the number of neurons that would show β-galactosidase activity at 21 DIV. We could not verify any significant change in terms of the number of X-GAL-positive neurons in cultures exposed chronically during 20 days to either drug, though neurons at 21 DIV show a relatively large number of X-GAL-positive neurons (Fig. 9A). Neither MDMA nor AMPH concentrations of 1, 10 or 100 μM could promote an increase in this marker of cellular senescence (Fig. 9B). Preliminary experiments were also done at 14 DIV, but the results were similar to the ones obtained at 21 DIV and no increase in cellular senescence could be found (data not shown).

**Morphological evaluation of neurons exposed chronically to 100 μM of AMPH or MDMA**

We evaluated microscopically the effects of drugs over time in neurons at different stages of maturity. The effects of ATS in long-term neuronal cultures were registered in microphotographs (Fig. 9C). Both AMPH and MDMA, at the concentration of 100 μM, promoted a pronounced cell loss at 8 DIV. Moreover, at later time-points, namely at 20 DIV, cortical cultures showed more signs of neurodegeneration after exposure to drugs when compared to controls, though degeneration in control cultures is at this stage already high (Fig. 9C).

**DISCUSSION**

The key findings of our study in cultured cortical neurons were: (1) long-term maintenance of primary cultures of the cortex was established up to 28 days, with cultures showing roughly doubling the percentage of astroglial cells from 8 to 21 DIV; (2) the implemented protocol of exposure assured that the drug concentration in neuronal medium did not decrease during the continuous exposure; (3) at 8 DIV, ATS promoted a significant change in the percentage of neurons and astroglial cells present in culture, promoting a global decrease in the number of both cells; (4) in cultures up to 14 DIV AMPH promoted earlier mitochondrial dysfunction and neuronal death than MDMA, and, for both drugs, caspase 3 activity increased only for drug concentrations above 100 μM; (5) AMPH, but not MDMA, elicited a decrease in intraneuronal GSHt levels at 8, 14 and 21 DIV; (6) glutamate receptor antagonists could neither ameliorate the mitochondrial dysfunction nor the cell death promoted by either drug at 14 DIV; (7) in long-term cultures, up to 21 and 28 DIV, concentrations equal to or lower than 10 μM had no effect in cellular viability, mitochondrial function or altered the glutathione status of neurons; (8) neither...
Table 2. Effect of glutamate antagonists against the cell death and mitochondrial dysfunction promoted by 200 μM of AMPH or MDMA

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ATS 200 μM</th>
<th>ATS + MK-801</th>
<th>ATS + CNSX</th>
<th>ATS + CNSX + MK-801</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMPH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of controls (MTT test)</td>
<td>100.0 ± 5.3</td>
<td>65.6 ± 11.4</td>
<td>69.0 ± 11.9</td>
<td>96.0 ± 8.2</td>
<td>96.0 ± 11.9</td>
</tr>
<tr>
<td>% of cell death (LDH assay)</td>
<td>100.0 ± 4.8</td>
<td>67.2 ± 6.0</td>
<td>64.5 ± 6.4</td>
<td>64.5 ± 6.4</td>
<td>64.5 ± 6.4</td>
</tr>
<tr>
<td><strong>MDMA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of controls (MTT test)</td>
<td>100.0 ± 5.4</td>
<td>67.3 ± 6.0</td>
<td>64.5 ± 6.4</td>
<td>64.5 ± 6.4</td>
<td>64.5 ± 6.4</td>
</tr>
<tr>
<td>% of cell death (LDH assay)</td>
<td>100.0 ± 4.8</td>
<td>67.2 ± 6.0</td>
<td>64.5 ± 6.4</td>
<td>64.5 ± 6.4</td>
<td>64.5 ± 6.4</td>
</tr>
</tbody>
</table>

Lack of protection afforded by glutamate antagonists against the cell death and mitochondrial dysfunction promoted by AMPH or MDMA (ATS – amphetamine-type psychostimulant). Cultures of cortical neurons were exposed continuously after the first day until 14 DIV to either AMPH or MDMA (200 μM). The glutamate antagonists MK-801 (1 μM) and/or CNQX (10 μM) were added to the cultures at 4 DIV and remained in the medium until 14 DIV for protection.

ANOVA (7, 176) = 32.63, (7, 134) = 34.83, (7, 128) = 63.3, (7, 134) = 87.85,
Bonferroni post hoc test.

***p < 0.0001 for the MTT assay, and
***p < 0.0001 for the LDH assay; in the case of MDMA

AMPH nor MDMA increased the number of senescent neurons counted as X-GAL-positive staining in long-term exposure at 21 DIV.

In the present paper, we describe the effects of chronic and continuous exposure to the ATS, AMPH or MDMA, on cultured neurons from the rat cortex. When using long-term incubation of neuronal cultures with drugs, one might speculate whether within the cell culture medium the concentration of drugs would remain stable after several days in an incubator at 37 °C, as these conditions would favor chemical degradation of the drugs. In fact using our protocol, we could maintain the concentration of AMPH and MDMA within the expected concentration range between the 8th and the 14th day of incubation, without any signs of chemical degradation of the drugs. Instead, for the 200 μM concentration of either AMPH or MDMA there was a slight, but statistically significant, increase in the concentration measured at 14 DIV when compared to the one measured at 8 DIV. This is certainly due to medium evaporation from 8 to 14 DIV, where cells had to wait 6 days without feeding. Changes were not significant for concentrations equal or below 100 μM of either drug at the evaluated time-points. In cells exposed up to 28 DIV feeding occurred every 4th day rendering more unlikely the changes due to evaporation. Therefore, with our protocol of chronic exposure to drugs, we assume that concentrations equal or below 100 μM of either drug will remain within the expected range up to 28 DIV. Importantly, given that we could not detect MDMA metabolites, it seems reasonable to assume that metabolism of both drugs does not seem to occur in these cultures. The absence of metabolites correlates well with the low metabolic ability of neurons given the minor cytochrome P450 content (Hedlund et al., 2001). Therefore the metabolites of drugs do not seem to play a role in the effects observed.

Generally, cell culture studies with ATS are designed to study their acute effects on neuronal cells, with several parameters being assessed 24–96 h after exposure (Stumm et al., 1999; Jimenez et al., 2004; Capela et al., 2006b, 2013; Wang et al., 2008; Ruscher et al., 2011). Overall these studies use concentrations of ATS ranging from 100 μM up to 5 mM, which provide limitations regarding the extrapolation of data to the in vivo situation of chronic exposure. From the above mentioned studies, it was possible to conclude that AMPH, MDMA or methamphetamine promote neuronal apoptosis in a concentration-dependent manner, accompanied by caspase 3 activation. Indeed it seems that mitochondrial death pathways are involved in neurodegeneration since cytochrome c release (Jimenez et al., 2004) and apoptosis inducing factor cleavage (Capela et al., 2013) were reported. In our laboratory, we have performed studies with neuronal cortical and hippocampal cultures that were treated after the 8 or 10 DIV, respectively with MDMA in a concentration range 100–800 μM, a single application without feeding for the following 48 h (Capela et al., 2006b, 2007a, 2013). These studies were intended to evaluate the result of an acute exposure to high levels of MDMA, correlating more with acute exposure by the...
abuser. These studies fail to mimic the chronic exposure to drugs that occurs after continuous therapeutic or illegal use.

Our present protocol reflects a paradigm of chronic long-term exposure to the drugs using a neuronal culture system. Our results clearly show that chronic exposure of neurons to concentrations equal or above 100 μM of ATS up to 27 DIV (meaning 27 days of exposure to drugs) can promote significant mitochondrial dysfunction and elicit neuronal death. Additionally, long-term exposure to 1 or 10 μM of AMPH or MDMA did not promote neuronal death or altered the neuronal glutathione status. These results demonstrate the need for a certain threshold level of ATS to trigger neurotoxic events related to neuronal damage. Importantly, shorter times of exposure (4 DIV) to AMPH were sufficient to trigger neuronal death compared with MDMA, while at later time-points (8 and 14 DIV) drugs induced a similar cell death. Noteworthy, the concentrations of 1 or 10 μM of AMPH promoted a transient mitochondrial dysfunction at 8 DIV, which was not accompanied by an increase in cell death and was no longer seen at 14 DIV. Interestingly, neuronal death was more pronounced at 8 DIV for both drugs (100 and

Fig. 7. AMPH- and MDMA-induced mitochondrial dysfunction, cell death and total glutathione to cortical neurons at 21 DIV. Cultures of cortical neurons were exposed continuously after the first day until 21 DIV to AMPH or MDMA (1, 10 and 100 μM). (A, B) Mitochondrial dysfunction evaluated by the MTT assay promoted by AMPH or MDMA, respectively (three different and independent experiments, each experiment having six different culture wells per condition). (C, D) Percentage of cell death evaluated by the LDH assay after AMPH or MDMA exposure, respectively (four different and independent experiments, each experiment having six different culture wells per condition). (E, F) Total glutathione (GSHt) levels in neuronal cultures after AMPH or MDMA exposure, respectively (four different and independent experiments, each experiment having two different culture wells per condition). Statistical analysis conducted by the Kruskal–Wallis test (A, Kruskal–Wallis statistic = 32.56, p < 0.0001; B, Kruskal–Wallis statistic = 16.19, p < 0.001; C, Kruskal–Wallis statistic = 17.67, p < 0.001; D, Kruskal–Wallis statistic = 24.48, p < 0.0001; E, Kruskal–Wallis statistic = 8.45, p < 0.05; F, Kruskal–Wallis statistic = 0.78, p > 0.05), followed by Dunn’s post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001 concentration vs. control; #p < 0.05, ###p < 0.001 concentration vs. 1 μM; $$ p < 0.01, $$$ p < 0.001 concentration vs. 10 μM).
200 µM) than at 14 DIV, and at this later time-point no neuronal death could be seen for the 100 µM concentration of either drug. In accordance with the previous results, the increase of caspase 3 activity was only seen at 8 DIV for the 200 µM concentration of both drugs, but was lost at 14 DIV. It is reasonable to assume that the increase in necrosis (seen by LDH leakage) or other forms of death at 14 DIV overcame the apoptotic signs elicited by MDMA or AMPH at this time-point. Moreover, caspase 3 activity in control neurons almost doubled from 8 to 14 DIV, as a sign of ongoing natural degeneration resulting from long-term maintenance in culture. At latter time-points of exposure, 21 and 28 DIV, neither AMPH nor MDMA could promote an increase in the percentage of cell death, but were able to elicit a significant mitochondrial dysfunction for 100 µM of both drugs at 28 DIV. In fact, at 21 DIV the percentage of cell death was lower for 100 µM of both drugs, most likely reflecting the lower release of LDH due to less neuronal necrotic cell death. Additionally, 100 µM of MDMA promoted an increase in MTT metabolism at 21 DIV, most likely as a result of an increase in mitochondrial complexes activity, since MTT is reduced by complex I and II of the mitochondrial respiratory chain (Berridge et al., 2005; Costa et al., 2009b). Overall, neuronal cultures at earlier time-points namely between 4 and 8 DIV showed more significant neurotoxicity toward ATS than at the latter time-points 21 or even 28 DIV. Possibly, the inability of the drugs to affect neurons after long-term treatment, might indicate that the most sensitive neurons degenerated early in the treatment, and at latter time-points 21 and 28 DIV only ATS-insensitive neurons remain. From the above mentioned, it is clear that the different developmental stages of neuronal culture, containing a differential abundance of neurons and astroglial cells, strongly influence the neurotoxicity promoted by ATS.

It is widely acknowledged that oxidative stress is known to play an important role in ATS neurotoxicity, as both in vivo (Pereira et al., 2006; Wan et al., 2006) and in vitro (Jimenez et al., 2004; Capela et al., 2006b, 2007a). We have previously reported that MDMA (concentration range of 200–800 µM) treatment in an acute exposure model, promoted a reduction in GSHt levels in cortical neuronal cultures after 48 h of exposure (Capela et al., 2007a). In the present model of chronic exposure, only AMPH could promote significant decreases in intracellular GSHt levels, at 8 and 14 DIV for 200 µM and at 21 DIV for the 100 µM concentration. Interestingly, only the concentration of 100 µM at 8 DIV promoted a significant alteration in the intracellular GSSG levels, with a decrease that may be explained by GSSG efflux from the cells (Costa et al., 2009a). The decrease of intracellular GSHt promoted by 200 µM of AMPH was not accompanied by an increase in GSSG levels, which remained equal to control levels at 8 and 14 DIV. This means that

Fig. 8. AMPH- and MDMA-induced mitochondrial dysfunction and cell death to cortical neurons at 28 DIV. Cultures of cortical neurons were exposed continuously after the first day until 28 DIV to AMPH or MDMA (1, 10 and 100 µM). (A, B) Mitochondrial dysfunction evaluated by the MTT assay promoted by AMPH or MDMA, respectively. (C, D) Percentage of cell death evaluated by the LDH assay after AMPH or MDMA exposure, respectively. Results were obtained from four different and independent experiments, each experiment having six different culture wells per condition. Statistical analysis conducted by the Kruskal–Wallis test (A, Kruskal–Wallis statistic = 49.75, \( p < 0.0001 \); B, Kruskal–Wallis statistic = 37.66, \( p < 0.0001 \); C, Kruskal–Wallis statistic = 3.21, \( p > 0.05 \); D, Kruskal–Wallis statistic = 1.9, \( p > 0.05 \)) followed by Dunn’s post hoc test (**\( p < 0.001 \) concentration vs. control; ***\( p < 0.001 \) 100 µM vs. lower concentrations).
the reduced glutathione/GSSG ratio is decreased in comparison to controls revealing a clear decrease in the intracellular antioxidant status. MDMA did not interfere with the intracellular levels of GSHt or GSSG at any concentration or time-point tested. It seems that in our model oxidative stress does not seem to play a significant role in the effects of MDMA at any concentration tested, as well as in the case of concentrations equal to or lower than 10 \( \mu \text{M} \) of AMPH. MDMA metabolism seems to be the major cause of oxidative stress to neurons, as shown in our studies that applied MDMA metabolites directly to cultured neurons (Capela et al., 2006a, 2007b) and since, in our model, the metabolites were not detected, it is expected that neurons exposed to MDMA do not show any sign of oxidative stress, even in long-term exposures up to 28 DIV. In a previous study, AMPH showed a significantly higher ability to promote a pro-oxidant status seems a plausible explanation why, in our model, AMPH promoted a reduction in cellular viability at earlier time-points than MDMA.

Differentiation of neurons and their maintenance in culture requires glutamate (Brewer, 1995), but, at the same time, the role of glutamate excitotoxicity is very well-known in the neuronal death seen in hypoxia–ischemia, epilepsy or degenerative diseases (Lau and Tymianski, 2010). Using cortical neuronal culture, we have previously shown that MK-801 could partially prevent the neurotoxicity afforded by 400 \( \mu \text{M} \) of MDMA after 48 h acute exposure (Capela et al., 2006b). We sought to test the putative protection of glutamate antagonists co-incubating them with ATS. When co-incubated with 200 \( \mu \text{M} \) of AMPH or MDMA at 4 DIV, neither (+) MK-801, a selective antagonist of NMDA receptors, nor CNQX, a mixed antagonist of AMPA/kainate, prevented either the mitochondrial dysfunction or the neuronal death seen at 14 DIV. Additionally, when virtually the three glutamate receptors, NMDA,
AMPAs, and kainate, were blocked by the co-exposure with both antagonists, no protection was verified. These results clearly show that, in our exposure model, the neurotoxicity observed for the higher concentrations tested was not dependent on glutamate excitotoxicity. Importantly, CNQX per se was able to decrease the ongoing neuronal death in cultured cells at 14 DIV, but not the combination of both CNQX and MK-801. This differential effect argues for diverse roles of the glutamate receptors in the ongoing neurodegeneration in culture. In another study, using an acute exposure of cerebellar granule neurons to MDMA, at concentrations ranging from 1 to 5 mM for 48 h, neither MK-801 nor CNQX could prevent neurotoxicity (Jimenez et al., 2004). On the other hand, in vivo studies with rodents have shown that NDMA receptor antagonists can avoid the neurotoxic events resulting from acute exposure to ATS (Wan et al., 2000; Chipana et al., 2008b). However, until this present work there were no publications showing whether glutamate antagonists could prevent the neurotoxicity resulting from chronic or long-term exposure to ATS in cell culture or in vivo settings.

In this study, we demonstrated that, subsequent to their differentiation, primary cortical neurons in prolonged culture develop characteristics of senescence. First of all, the cells developed morphological changes characteristic of aged neurons, namely adoption of enlarged cell body and long neuritic processes. In long-term cultures at 21 or 28 DIV, neurons were embedded within an elaborate plexus of axons, dendrites, and synapses, which are characteristics for the senescent phenotype (Lesuisse and Martin, 2002). Second, the levels of GSHt decreased in long-term cultures. GSHt levels peaked at 8 DIV and presented a minor decline at 14 DIV, while cultures at 21 DIV showed one third of the levels presented at 8 DIV. The decrease in GSHt levels in several rat brain areas accompanied by a decrease in γ-glutamylcysteine synthetase, the rate-limiting enzyme in de novo synthesis, has been reported to occur during aging (Liu, 2002). Third, we also evaluated the levels of β-galactosidase activity, detected by histochemical staining, to identify senescent neurons (Chernova et al., 2006), and verified that at 21 DIV control cultures exhibited approximately half of neurons positive for that staining. Chernova et al. (2006) clearly showed that the percent of β-galactosidase activity in mouse cortical neurons increased with the time of culture, an index of cultures aging. We could not measure any significant change in terms of β-galactosidase activity, as the number of X-GAL-positive neurons, in cultures exposed chronically up to 21 DIV to ATS, thus being this marker of cellular senescence not affected by ATS exposure. In accordance with our present findings, Adori and co-workers have shown that intermittent MDMA administration (15 mg/kg, i.e. every 7th day for 4 weeks) or single-dose treatment (15 mg/kg, i.p.), to adolescent/young adult male Dark Agouti rats, could not induce accelerated aging processes or neurodegeneration in senescence when rats were evaluated 20 months following treatment (Adori et al., 2011).

Our cultures show at 21 DIV almost the double of the percentage of astroglial cells when compared to cultures at 8 DIV. Older cultures have a higher percentage of astroglial cells, but this does not correspond to an increase in the number of astroglial cells per mm², as there was a modest decrease in the number of GFAP-positive cells from 8 to 21 DIV in control cultures. Thus, there seems to be no further proliferation of astroglial cells after 8 DIV. Moreover, older cultures have roughly half the neurons of younger cultures at 8 DIV, reflecting the naturally ongoing degeneration of neurons with the time of culture. These data may correlate and reflect the high caspase 3 activity at 14 DIV of control cultures, indicating that the loss of cells occurs mainly by apoptosis. ATS have an impact on the relative percentage of neurons and astrocytes present in culture. At 8 DIV, both drugs promoted a significant change in the percentage of neurons and astrocytes present in culture, promoting a global decrease in the number of both cells, but mainly astroglial cells. Thus, cultures with different stages of maturity present a diverse relative abundance of neurons versus astroglial cells, with impact in the outcome following exposure to ATS. The difference in the population can be another reason for the lower sensitivity to the toxicity of ATS at later time-points 21 or 28 DIV versus 8 DIV. Therefore, the maturity stage of the culture and the role of astrocytes in long-term cultures should not be neglected.

Other works conducted in primary rat cortical neurons, using a density at seeding of 0.5 × 10⁵ cells/cm² and Neurobasal medium supplemented with B27, reported that the number of astrocytes can reach 35–40% at day 16 as compared to the number of neurons (Costantini et al., 2010). Even though the percentage of astrocytes was roughly stabilized after 16 days in culture, western blot analysis of the astrocytic protein GFAP never stopped increasing until the 28 day in culture, meaning that astrocytes stopped dividing but continued to develop (Costantini et al., 2010). Using mouse cortical neuronal cultures, seeded at a density of 1 × 10⁵ cells/cm² and cultivated with Neurobasal medium supplemented with B27, another laboratory reported that GFAP levels evaluated by western blot remained constant from 20 to 60 DIV (Lesuisse and Martin, 2002). In our study, we used a cell density of 1.5 × 10⁵ cells/cm², which is higher than the two previously mentioned studies, and with impact in the relative abundance of astroglial cells.

Current cell culture paradigms that study ATS toxicity are designed to access the acute toxicity obtained after short-term exposure. Herein, we describe for the first time a model of chronic exposure to ATS using primary cortical neurons, which assures the maintenance of ATS’ concentration within the neuronal medium during 27 days in culture (from 1 to 28 DIV). Additionally, we performed chronic exposure of neurons using low micromolar concentrations, unlike the majority of acute paradigms that use up to millimolar concentrations. The concentrations used in our model were proven to be achieved in vivo and have significance to the human situation. A neuronal culture model that attempts to mimic chronic exposure to ATS is of great value, for the prediction of the long-term consequences of drug abuse, including those associated with neuronal development and aging. With our model, using concentrations equal...
to or below 10 µM, which do not promote cell loss in incubations up to 28 DIV, several events unrelated to neuronal death (intracellular signaling, receptor changes, neurite outgrowth) can be studied without the confounding factor of neuronal death.

CONCLUSION

Long-term treatment of neuronal cultures mimicking chronic exposure to ATS is of great value to predict the consequences related to drug abuse associated with development and aging. Further studies using cultured neuronal models of chronic exposure to low concentrations of ATS as the ones attained in vivo are required to get further insights into the consequences of chronic drug abuse.

Acknowledgments—This work was supported by “Fundação para a Ciência e Tecnologia” (FCT), Portugal (Project PTDC/SAU-FCF/102958/2008), under the frame of “Programa Operacional Temático Factores de Competitividade (COMPETE) do Quadro Comunitário de Apoio III” and “Fundo Comunitário Europeu (FEDER)” (FCOMP-01-0124-FEDER-011079), VMC and JPC acknowledge FCT for their Post-doc grants (SFRH/BPD/63746/2009 and SFRH/BPD/30776/2006, respectively).

REFERENCES


Law MYL, Moody DE (2000) Urinary excretion of 4-hydroxy-phenyl- 
N-tert-butyl nitrone and 


Pereira FC, Lourenço ES, Borges F, Morgadinho T, Ribeiro CF, Macedo TR, Aiú SF (2006) Single or multiple injections of methamphetamine increased dopamine turnover but did not decrease tyrosine hydroxylase levels or cleave caspase-3 in caudate-putamen. Synapse 60:185–193.


