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Methamphetamine produces neuronal inclusions in the nigrostriatal system and in PC12 cells

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Abstract

Mice treated with the psychostimulant methamphetamine (MA) showed the appearance of intracellular inclusions in the nucleus of medium sized striatal neurones and cytoplasm of neurones of the substantia nigra pars compacta but not in the frontal cortex. All inclusions contained ubiquitin, the ubiquitin activating enzyme (E1), the ubiquitin protein ligase (E3-like, parkin), low and high molecular weight heat shock proteins (HSP 40 and HSP 70). Inclusions found in nigral neurones stained for α-synuclein, a proteic hallmark of Lewy bodies that are frequently observed in Parkinson's disease and other degenerative disorders. However, differing from classic Lewy bodies, MA-induced neuronal inclusions appeared as multilamellar bodies resembling autophagic granules. Methamphetamine reproduced this effect in cultured PC12 cells, which offered the advantage of a simple cellular model for the study of the molecular determinants of neuronal inclusions. PC12 inclusions, similar to those observed in nigral neurones, were exclusively localized in the cytoplasm and stained for α-synuclein. Time-dependent experiments showed that inclusions underwent a progressive fusion of the external membranes and developed an electrodense core. Inhibition of dopamine synthesis by α-methyl-*p*-tyrosine (αMpT), or administering the antioxidant *S*-apomorphine largely attenuated the formation of inclusions in PC12 cells exposed to MA. Inclusions were again observed when αMpT-treated cells were loaded with L-DOPA, which restored intracellular dopamine levels.

Keywords: α-synuclein, methamphetamine, neuronal inclusions, parkin, proteasome, ubiquitin.

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Methamphetamine (MA) neurotoxicity is becoming increasingly important because of the wide abuse of this drug in USA and Europe (Lukas 1997; National Institute on Drug Abuse. Methamphetamine abuse alert 1999). Both MA and its derivative, 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy'), are toxic to monoaminergic nerve terminals as a result of free radical formation (Seiden and Ricaurte 1987; De Vito and Wagner 1989; Cadet *et al*. 1994; Giovanni *et al*. 1995; Kalant 2001). MA treatment induces nigro-striatal damage in experimental animals and humans (Seiden *et al*. 1975; Ricaurte *et al*. 1982; Wilson *et al*. 1996), and therefore it is considered as a model of drug-induced parkinsonism (Gerlach and Riederer 1996). Many neurodegenerative disorders are characterized by the presence of neuronal inclusions. In Parkinsons's disease (PD) these inclusions contain various components of the ubiquitin-proteasome pathway, which operates as an intracellular protein-clearing system (Bence *et al*. 2001; Chung *et al*. 2001a). In PD, degeneration of nigral dopaminergic

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Abbreviations used: αMpT, α-methyl-*p*-tyrosine; DA, dopamine; DAT, dopamine transporter; 2,3-DHBA, 2,3-dihydroxybenzoic acid; DOPAC, dihydroxyphenylacetic acid; GFAP, glial fibrillary acidic protein; H&E, haematoxylin and eosin; 5HT, serotonin; HSP, heat shock protein; HVA, homovanillic acid; i.p., intraperitoneally; MA, methamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; PBS, phosphate-buffered saline; PD, Parkinson's disease; SNpc, pars compacta of substantia nigra; TH, tyrosine hydroxylase.

neurones is accompanied by the formation of inclusions named Lewy bodies, which contain α-synuclein and specific components of the ubiquitin-proteasome pathway, such as parkin and the ubiquitin C-terminal hydrolase L1 (Lowe *et al*. 1990; Spillantini *et al*. 1997; Schlossmacher *et al*. 2002). Mutations of these proteins are associated with inherited PD (Polymeropoulos *et al*. 1997; Kitada *et al*. 1998; Leroy *et al*. 1998; Mouradian 2002). Interestingly, MA has been shown to induce intracellular multilamellar bodies in the cytoplasm of cultured midbrain dopaminergic neurones (Cubells *et al*. 1994; Larsen *et al*. 2002). These inclusions are morphologically different from classic Lewy bodies (which are not membrane-delimited), but, nevertheless, their formation can be involved in the pathophysiology of MA-induced parkinsonism. We now report that MA treatment induces the formation of neuronal inclusions also *in vivo*, both in nigral and striatal neurones. In addition, we analyzed cortical neurones and we tried to reproduce this phenomenon in cultured PC12 cells, a homogenous cell population that allowed use to examine the development of neuronal inclusions as a function of time and the role of endogenous dopamine (DA) in their formation.

Materials and methods

Animals

Male C57BL/6J, 9–10 weeks old, obtained from Charles River (Calco, Italy) were kept at constant room temperature (21°C), number of mice per cage ($N = 10$) and the size of the cage (11 × 10 cm wide and 15 cm high). Mice were handled in accordance with the Policy on Human Care and Use of Laboratory Animals of the United States Public Health Service. All the experiments were approved by the local Ethical Committee for Animal Studies.

Drug treatments

Neurotoxicity experiments were carried out as previously described (Battaglia *et al*. 2002). MA hydrochloride (Sigma Chemical Co., St. Louis, MO, USA) was administered intraperitoneally (i.p.) at a dose of 5 mg/kg × 4, 2 h apart. This dosage of MA was based on pilot dose–response experiments and previous studies (Fornai *et al*. 1999), and it was chosen in order to produce a marked neurotoxic effect to the dopaminergic nigrostriatal pathway in the absence of nigral cell loss. Each treatment group was composed of 10 animals. Seven days after the administration of MA or saline, mice were killed by decapitation, and the brains were dissected for the detection of striatal monoamine levels (Battaglia *et al*. 2002), and for immunocytochemical analysis (see below).

Cell culture

The rat pheochromocytoma cell line PC12, was obtained from the American Type Culture Collection and grown in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, penicillin (50 IU/mL) and streptomycin (50 mg/mL). Cells in log phase were used for the experiments. The dose of MA (1 μM) was selected based on results of the dose–response study (see Fig. 5j,k) in order to get the highest amount of intracellular inclusions in the absence of cell loss. In fact, when increasing the dose of MA at doses ranging between 10 and 100 μM, the cell pellet contained less cells which were disrupted and at above 100 μM of MA there was a massive cell loss (i.e. we could not find intact PC 12 cells in the pellet). Further experiments were carried out exposing cells to α-methyl-*p*-tyrosine (αMpT, 1 mM, Sigma, St Louis, MO, USA). Moreover, we supplied methyl-L-DOPA hydrochloride (500 μM; Sigma) as a DA precursor or the *S*-enantiomer of apomorphine, which is an antioxidant iron-chelating agent, nonactive on DA receptors. The doses of *S*-apomorphine (Sigma) ranged between 0.1 and 10 μM based on the present study and previous experiment on the antioxidant effects on PC12 cells (Gassen *et al*. 1998). We also combined the antioxidant *S*-apomorphine with the TH inhibitor αMpT to evaluate whether a combination of these drugs might be more efficacious in preventing inclusion formation. Drugs were dissolved directly in the culture medium.

Immunocytochemistry and immunoblots

Immunocytochemical analysis (peroxidase or fluorochrome-revealed primary antibodies, Vector Laboratories, Burlingame, CA, USA) was performed as previously described (Fornai *et al*. 2001). Twenty-micrometre thick sections of striatum and substantia nigra were obtained at cryostat, whereas slides of PC12 were obtained by spinning the cell at 12 000 × *g* for 10 min (Cytospin 3, Shandon) over the slides.

The sources of the antibodies were: tyrosin hydroxylase (TH), ubiquitin, heat shock protein (HSP) 70 and HSP 40 from Sigma Chemical Co.; DA transporter (DAT), glial fibrillary acidic protein (GFAP), ubiquitin protein ligase (E3-like parkin) and ubiquitin activating enzyme (E1) from Chemicon International (Temecula, CA, USA); α-synuclein was provided by Oliver Schlueter and Thomas C. Südhof. For single immunolabelling studies, the brain sections were incubated with primary antibody, overnight at 4°C or 2 h at room temperature, followed by 1-h incubation with secondary antibody (1 : 50). All primary antibodies were used diluted 1 : 1000, except for α-synuclein and parkin antibodies which were diluted 1 : 500. The samples were analyzed by Wetzlar Orthoplan (Leitz) light microscope and Ultraview Confocal Imaging System, Perkin-Elmer confocal microscope. For confocal microscopy, 10 groups of optical Z-section serial slices from each experiment were taken with 0.5 μm Z-steps. Densitometric analysis of TH immunostaining (NIH Image Analysis) was carried out with the purpose to have a semiquantitative estimation of striatal DA terminals.

Western blots were performed as previously described (Iacovelli *et al*. 2001), using the anti-ubiquitin antibodies diluted at 1 : 1000.

Electron microscopy

Mice were perfused and brains were maintained *in situ* immersed in fixative solution [2% paraformaldehyde/0.1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4] overnight at 4°C and then removed from the skull. Caudate-putamen and substantia nigra were dissected and postfixed in 1% OsO_4 in buffered solution, dehydrated in ethanol and embedded in Epon-araldite. Electron microscopy on PC12 cells was carried out on cellular pellets. For plane electron microscopy cell pellets were fixed in 3% glutaraldehyde in 0.1 M PBS, pH 7.4, and postfixed in 1% OsO_4 buffered

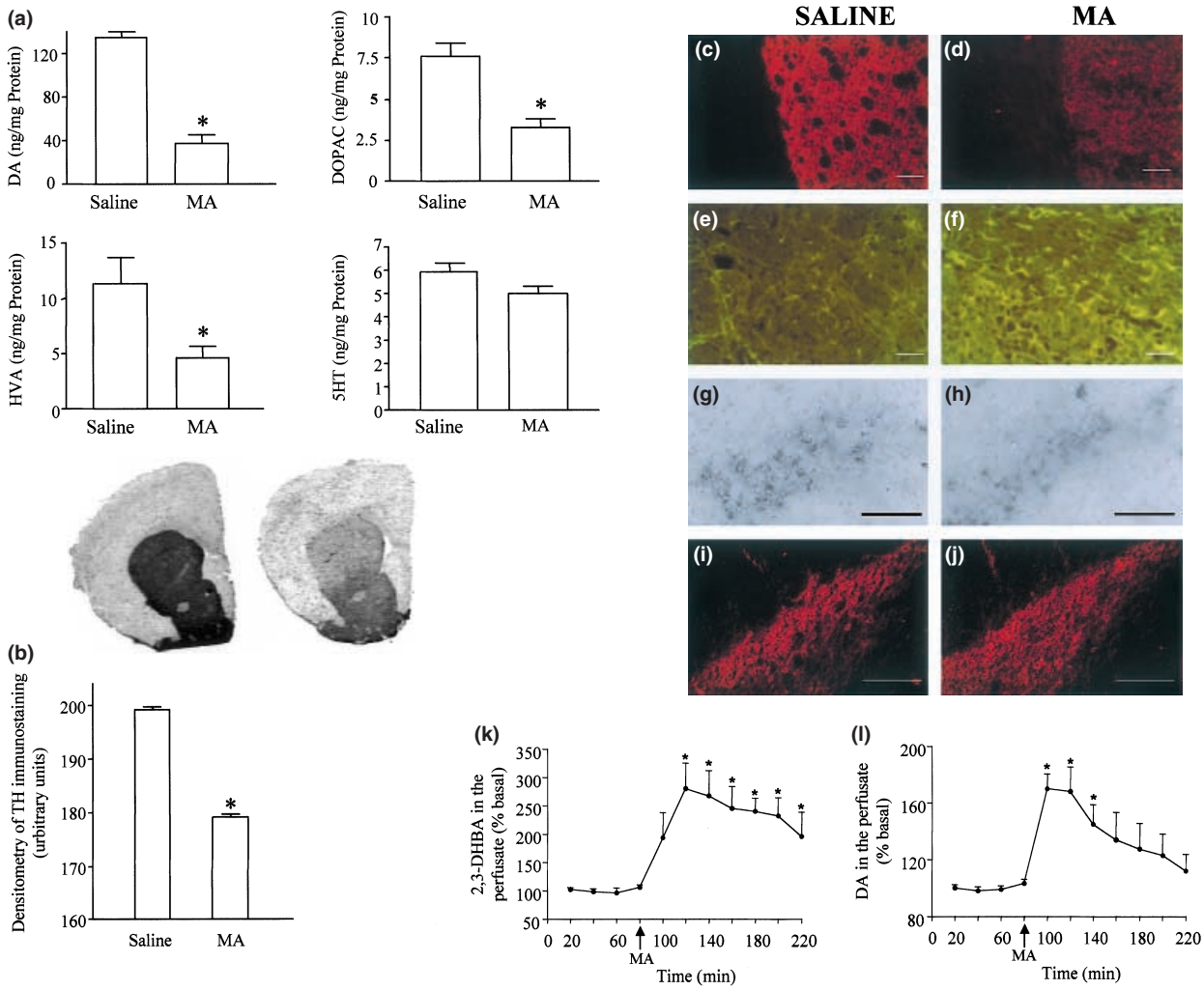

Fig. 1 MA treatment induces nigro-striatal damage in mice. (a) Striatal DA, DOPAC, HVA and serotonin (5HT) levels in mice injected with saline or MA. Values are means ± SEM of 10 determinations. *$p < 0.05$ (Student's *t*-test) versus saline. (b) Representative immunohistochemical analysis of striatal TH in saline or MA-treated mice. Densitometric analysis was performed to obtain a semiquantitative estimation on comparable sections from between five and six mice per group. *$p < 0.05$ (Student's *t*-test) versus saline. Immunofluorescent analysis of striatal DAT (c,d) and GFAP (e,f) in saline and MA-treated mice, respectively, is shown. In MA-treated mice there is a marked loss of DAT immunostaining (d), while an increased immunofluorescence for the glial marker GFAP can be observed (f). In contrast no difference between control and MA compared were evident at nigral level as visualized by representative micrographs obtained using antibodies against TH (g,h, respectively) and DAT (i,j, respectively). Scale bars = 100 μm (k) Formation of radical oxygen species induced by MA in the caudate nucleus, as assessed by the conversion of salicylate into 2,3-DHBA in freely moving mice. (l) increase of striatal extracellular DA levels measured by microdialysis following MA. Values are means ± SEM of six determinations. *$p < 0.05$ (one-way ANOVA + Sheffe's post hoc test) versus saline.

solution. Then, they were prestained en bloc with a solution of 5% uranyl acetate in 30% ethanol, dehydrated and embedded in Epon-araldite. Ultrathin sections of both brain specimens and PC12 cells were stained with uranyl acetate and lead citrate, and finally examined at Jeol Jem 100SX transmission electron microscope (Jeol, Tokyo, Japan). For immunoelectron microscopy 50 nm thick sections were stained for gold-conjugated revealed primary antibodies previous listed, used at 1 : 100 dilution; gold particles ranged 10–15 nm in diameter. For immunolabelling PC12 cells were not prestained en bloc.

Count of whorls

Measurement of nigral, striatal, cortical and PC12 whorls was carried out by counting the number of either cytosolic or nuclear (striatum) whorls out of the total number of neurones under observation. *In vivo*, for each animal ($N = 10$) from each different group we chose randomly two tissue blocks dissected from the dorsal, ventral striatum (consisting of nucleus accumbens), frontal cortex and substantia nigra. From each block we got 10 grids, every grid containing several non-serial slices in order to observe 10 different neurones and count a final number of 2000 cells.

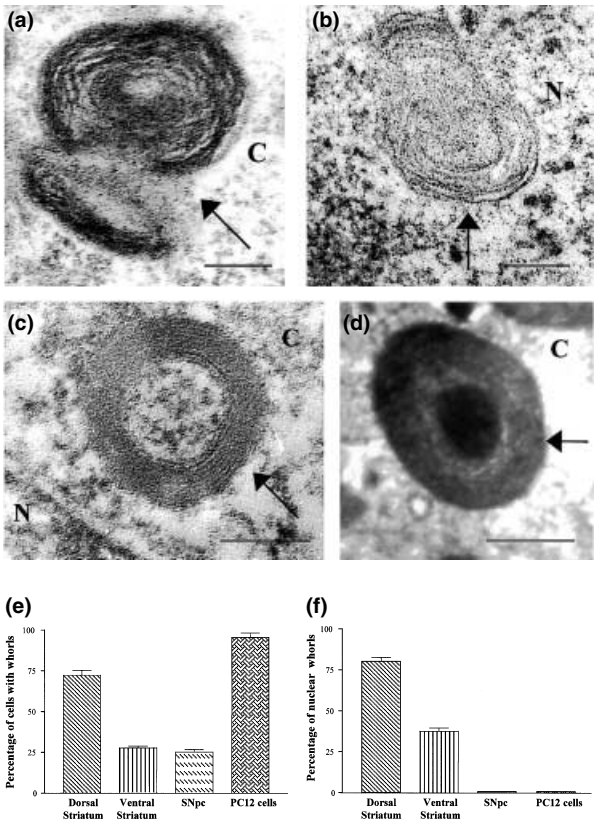

Fig. 2 Inclusion bodies in striatal medium size neurones and nigral neurones of MA-treated mice. Ultrastructural analysis of intraneuronal inclusions shows the presence of membranous multilayer whorls in the cytoplasm (a) and nucleoplasm (b) of striatal neurones. In nigral neurones the inclusions are exclusively cytoplasmic, and they appear as membraneous whorls surrounding a pale amorphous (c) or dense (d) core. Bar graphs show the percentage of whorls-containing neurones (e) and the percentage of whorls found in the cell nuclei (f) in the striatum, pars compacta of substantia nigra (SNpc) and PC12 cells after treatment with MA. Values were obtained by an average of 20 000 cells for each brain area (2000 cells for each mouse, $N = 10$) and 5000 PC12 cells (1000 cells per dish $N = 5$ sister dishes). Each single inclusion is indicated by an arrow. C, cytoplasm; N, nucleus. Scale bars = 0.25 μm.

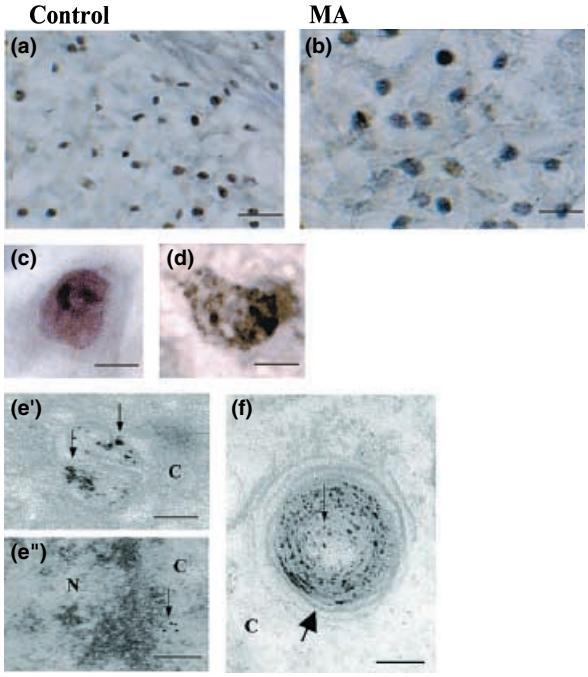

Fig. 3 Striatal intraneuronal inclusions in mice treated with MA. Ubiquitin immunostaining in striatal neurones from saline and MA-treated mice at low magnification light microscopy (a,b); oil immersion light microscopy (c,d) and electron microscopy (e′, e″,f), respectively, are shown. Note that ubiquitin is distributed in striatal neurones from control mice quite homogeneously at light (c) and electron (e′, immunoperoxidase light arrow; e″, immunogold light arrow) microscopy, but ubiquitin immunoperoxidase (light arrows) appears to be concentrated in a neuronal inclusion (thick arrow) of an MA-treated mice (f). Scale bars are: 25 μm (a,b); 5 μm (c,d) 0.15 μm (e′,e″,f).

Data concerning the percentage of whorls in the cytosol of striatal, nigral and PC12 cells or in the nucleus of striatal cells were compared using ANOVA with Sheffè's post hoc analysis. Null hypothesis (H_o) was rejected when $p < 0.05$. In fact, we could never find whorls in the nucleus of nigral or PC12 cells, where all inclusions localized in the cytoplasm. No whorl was found in the frontal cortex and whorls were virtually absent from any brain areas in mice injected with saline (two whorls out of 2000 counted cells in the dorsal striatum of one control mouse).

Microdialysis in freely moving mice

Microdialysis experiments were carried out as previously described (Battaglia *et al*. 2002). Briefly, mice were implanted with microdialysis intracerebral guides in the striatum (CMA/7 Guide Cannula, CMA/Microdialysis, Stockholm, Sweden), at the coordinates: 0.6 mm anterior to bregma, 1.7 mm lateral to the midline, 3.5–5.5 mm ventral from the surface of skull, according to the atlas of Franklin and Paxinos (1997). Concentric vertical microdialysis probes 2 mm long and 0.24 mm in outer diameter having a cuprophane membrane with a molecular cut-off of 6000 Da (CMA/7 Microdialysis Probe, CMA/Microdialysis, Stockholm, Sweden) were used. Sample fractions were collected before and after MA (5 mg/kg, i.p.) injection. Collected fractions were analyzed for assaying DA and metabolites and 2,3-dihydroxybenzoic acid (2,3-DHBA), a product of the reaction of salycilate (5 mM, added to ACSF) with hydroxyl radicals. After four sample fractions (used to determine the basal levels of monoamines and hydroxyl radicals), mice received methamphetamine to measure the simultaneous increase of 2,3-DHBA and monoamine levels, which were determined as previously described (Battaglia *et al*. 2002). In particular, monoamine levels were determined as reported for brain homogenate (see above).

Monoamine assay

Monoamines were assayed both in striatal homogenates and striatal dialysates. Striatal homogenates were obtained by sonication in 0.6 mL of ice-cold 0.1 M perchloric acid. Fifty microlitres of the homogenate were used for protein determination. The remaining

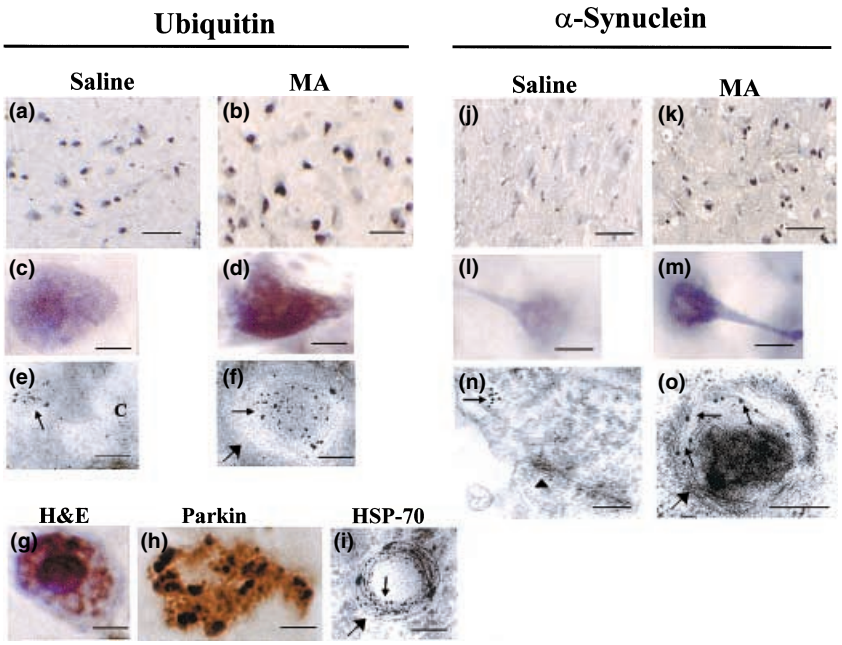

Fig. 4 Immunoreactive features of nigral intraneuronal inclusions in mice treated with MA. Ubiquitin immunoreactivity in nigral neurones is shown (a–f). Note that a difference between control and MA is already visible at low magnification (a,b, respectively); such a difference becomes more evident with oil immersion microscopy, where the control shows quite diffuse light immunostaining (c), while an MA-treated nigral cell possesses a marked staining (d). This is confirmed by immunoelectron microscopy (e,f), where scattered cytoplasmic immunoperoxidase staining (light arrow) within a control cell (e) contrasts with immunoperoxidase-labelled (light arrow) inclusions (thick arrow) evident as multilayer concentric whorls in MA-treated mice (f). Haematoxylin/eosin (H&E) staining (g) shows a pale eosinophilic cytoplasmic inclusion in a nigral neurone of an MA-treated mouse. Similarly (h) shows a nigral neurone from an MA-treated mouse densely stained for parkin. (i) Heat shock protein (HSP)-70 (light arrow) immunostained whorl (thick arrow) from a nigral neurone following MA. α-Synuclein immunostaining in nigral neurones is shown (j–n). At low magnification a slight difference can be observed between control (j) and MA-treated mice (k), where a more marked labelling is evident. This is more evident at oil immersion microscopy, where the control shows a pale and diffuse staining (l), while a cell from MA-treated mice (m) is markedly stained for α-synuclein. At an ultrastructural level, note the localization of α-synuclein (light arrow) close to a synapse (arrowhead) from a control mouse (n) and membranous whorls (thick arrow) positive for α-synuclein immunogold particles (light arrow) following MA (o) (diameter 10–15 nm). Scale bars are: 50 μm (a,b,j,k); 5 μm (c,d,g,l,m); 3 μm (h); 0.15 μm (e,f,i); 0.1 μm (n,o).

aliquots were centrifuged at 8000 × *g* for 10 min, and 20 μL of the supernatant were injected into an HPLC. Brain dialysates were collected every 20 min and 20 μL were injected into an HPLC. This was equipped with an autosampler 507, a programmable solvent module 126, an analytical C18 reverse-phase column (Beckmann Instruments, Fullerton, CA, USA) and a Coulochem II electrochemical detector (ESA Inc. Chelmsford, MA, USA). The holding potentials were set at +0.350 and −0.350 V for the detection of dopamine and metabolites. The mobile phase consisted of 80 mM sodium phosphate, 40 mM citric acid, 0.4 mM EDTA, 3 mM 1-heptanesulfonic acid and 12.5% methanol, brought to pH 2.75 with phosphoric acid (run under isocratic conditions, at 1 mL/min).

Results

Systemic administration of MA in mice (5 mg/kg, i.p., repeated four times at 2 h interval, $N = 10$) produced, 7 days later, a substantial lesion of striatal dopaminergic terminals associated with reactive gliosis, as assessed by measurements of striatal DA, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) levels (Fig. 1a), and by semiquantitative immunodetection of TH (Fig. 1b), high affinity DAT (Fig. 1c,d), and GFAP (Fig. 1e,f). Loss of striatal DA innervation occurred in the absence of significant nigral cell loss as shown by representative pictures (Fig. 1g,h for TH immunostaining and Fig. 1i,j for DAT immunostaining). Striatal neurotoxic effects can be ascribed to the formation of reactive oxygen species, which could be detected in the striatum of freely moving mice ($N = 12$) treated with MA (Fig. 1k) concomitant with increased extracellular DA levels measured by microdialysis (Fig. 1l).

Examination of striatal neurones of MA-treated mice by transmission electron microscopy revealed the presence of intracellular inclusions shaped as membranous whorls (Fig. 2a,b). Inclusions were prevalent in the dorsal striatum, and occurred more frequently in the nuclear region than in the cytoplasm (Fig. 2e,f), could never be detected in glial cells, and were found only exceptionally (two inclusions in

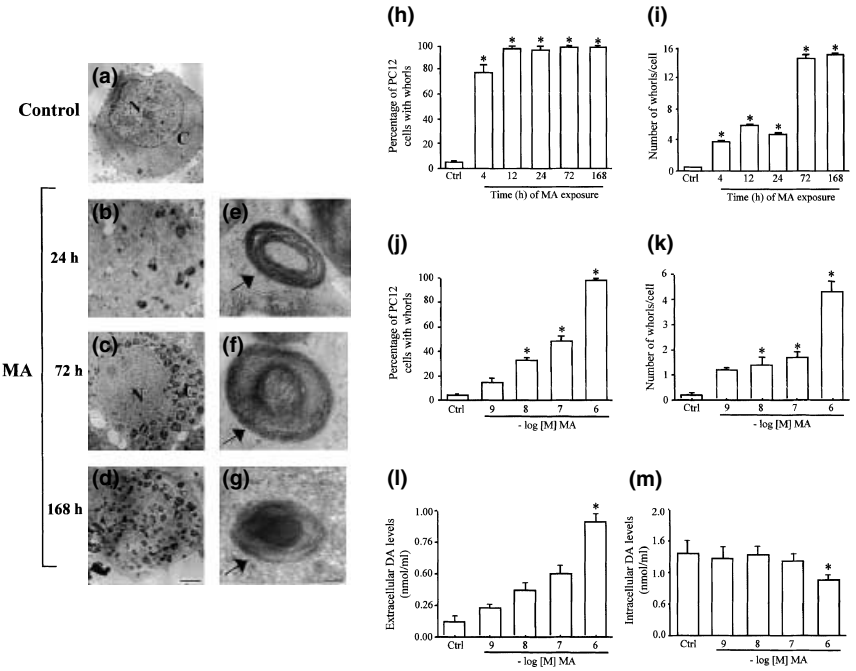

Fig. 5 Kinetics of inclusion body formation and DA release induced by MA in PC12 cells. Electron microscopic analysis of inclusion bodies in PC12 cells exposed to MA 1 μM for different time periods is shown. Note the increase in the number of whorls after MA exposure (b–d) compared with a control cell (a). At 72 and 168 h following MA exposure single PC12 cells (c and d, respectively) possess a cytoplasm completely filled of inclusions. These are magnified on the right, where single inclusions are shown (thick arrow). Methamphetamine exposure produces maturation of whorls showing an electron-dense core (e–g). Graphs show the number of cells with inclusions, and the concentration of inclusions per cell as a function of time of MA (1 μM) exposure (h,i, respectively). Dose–response graphs show that increasing the dose of MA leads to a higher number of cells with inclusions, and higher concentration of inclusions per cell (j,k, respectively), at the same time interval (24 h). Changes in extracellular (l) and intracellular (m) DA levels after 24 h of exposure to increasing doses of MA. Note that the increase in extracellular DA occurs for each increase in MA concentration (l), while only the highest dose of MA used (1 μM) produces a slight decrease of DA levels within the cells (m). Values are means ± SEM of 4–6 repeated determinations, each one carried out on 5000 cells (1000 per dish from five sister dishes). *$p < 0.05$ (one-way ANOVA + Sheffe's post hoc test) versus untreated PC12 cells (controls). N, nucleus; C, cytoplasm. Scale bars are: 3 μm (a,b,c,d); 0.3 μm (e,f,g).

one mouse) in control mice. Following MA administration, inclusions were also found within neurones of the pars compacta of the substantia nigra (Fig. 2c,d), where they were exclusively localized in the cytoplasm (Fig. 2e,f). Both striatal and nigral inclusions stained for components of the ubiquitin-proteasome system like ubiquitin (Figs 3d,f and 4d,f), the ubiquitin-activating enzyme (E1), the ubiquitin-ligase (E3-like, parkin, Fig. 4h), as well as low (HSP 40) and high (HSP 70, Fig. 4i) molecular weight heat shock proteins. However, only nigral inclusions, and not striatal inclusions, were immunoreactive for α-synuclein (Fig. 4m,o). By their ultrastructural and immunoreactive properties, neuronal inclusions were similar to the inclusions found in neurodegenerative disorders. As mentioned in the Methods section, occurrence of neuronal inclusions was evaluated by examining the presence of whorls at electron microscopy within 2000 cells from each brain area (dorsal striatum, ventral striatum, substantia nigra and all the layers of the frontal cortex) from each of the 10 mice treated with methamphetamine. This made 20 000 the total number of counted neurones for each brain area following methamphetamine. In addition, we found quite stable the number of inclusions when comparing different mice of the same group. This is likely to be due to the dose of methamphetamine used, which produced a maximal effect in term of inclusion formation. In fact, when rising two fold the dose of methamphetamine we could not increase the number of inclusions despite the neurotoxicity was more pronounced. As in *in vitro* experiments (see below) there is a plateau for inclusion formation. This is likely to account for the small variability between mice. In an attempt to reproduce these intracellular inclusions in a simple and homogeneous cellular model, we used undifferentiated PC12 cells, which derive from the neural crest and contain catecholamines. PC12 cells exposed to MA developed cytoplasmic multilamellar bodies that stained for all markers of the ubiquitin-proteasome pathway and for α-synuclein. These inclusions were similar to those found in nigral neurones of MA-treated mice. The effects of MA on PC12 cells were concentration-dependent, with almost the entire cell population developing the inclusions after 12–24 h

of exposure to 1 μM MA (Fig. 5). Higher doses of MA of up to 100 μM did not allow recovery of viable cells in the pellet and the cell remnants did not seem to contain whorls. The highest number of inclusions per single cell was detected after 72 h of exposure to MA 1 μM. Following the maturation of inclusions as a function of time, we observed that membranous whorls underwent a progressive fusion of the external membranes and developed an electron-dense core, similar to what observed for various shapes of nigral inclusions (Fig. 5e–g). Confocal microscopy analysis confirmed the formation of inclusions positively stained for ubiquitin and α-synuclein in MA-treated PC12 cells. Ubiquitin and α-synuclein were significantly colocalized within the inclusions (Fig. 6). The net amount of ubiquitin was not affected by MA treatment (Fig. 6e). We specifically examined whether intracellular catecholamines were essential for the formation of membranous whorls by treating PC12 cells with the TH inhibitor α-methyl-*p*-tyrosine (αMpT, 1 mM). This treatment, which substantially reduced intracellular DA levels, largely attenuated the formation of inclusions in response to MA. The effect of αMpT was reversed by L-DOPA (500 μM), which restored endogenous DA levels. L-DOPA also induced the formation of inclusions by itself and potentiated the effect of MA in the absence of αMpT (Table 1). We also found that the apomorphine enantiomer nonactive on DA receptors *S*-apomorphine (up to 10 μM) which possesses iron-chelating and antioxidant properties

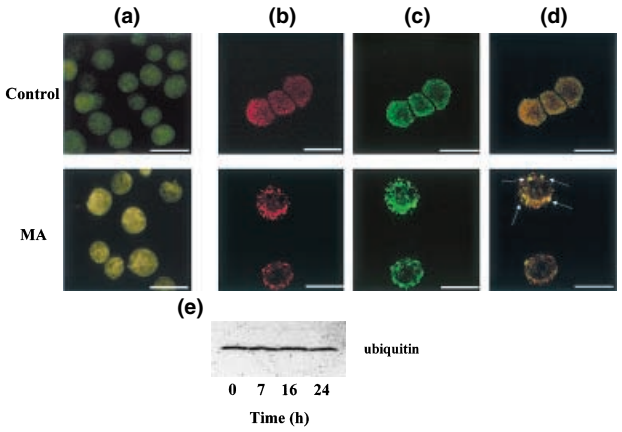

Fig. 6 Dynamic changes in ubiquitin and α-synuclein immunostaining in PC12 cells treated with MA. Immunofluorescent analysis of the ubiquitin-activating enzyme E1 in PC12 cells is shown in (a). Confocal microscopy analysis of ubiquitin (red) and α-synuclein (green) in PC12 cells is shown in (b) and (c), respectively. While control cells (upper line) show a diffuse staining, MA-treated cells (lower line) possess intense and localized stained inclusions. A high degree of colocalization concerning ubiquitin and α-synuclein (yellow) is shown in (d), where white arrows point to colocalization; scale bars = 10 μm. Western blot analysis of ubiquitin in PC12 cells exposed to 1 μM MA for different times (e). In (a–d), cultures were exposed to 1 μM MA for 24 h.

Table 1 Dopamine depletion reduces the formation of inclusion bodies induced by MA (1 μM, 24 h of exposure) in PC12 cells

Treatment	% MA-induced whorls/cell
Control	5.8 ± 0.7
αMpT	7.2 ± 1.2
L-DOPA	53.7 ± 9.5*
αMpT + L-DOPA	32.0 ± 5.7*
MA	100 ± 7.7
MA + αMpT	44.9 ± 8.0†
MA + L-DOPA	134.2 ± 9.7†
MA + αMpT + L-DOPA	106.4 ± 8.7

Values are mean ± SEM of 6–8 determinations. $p < 0.05$ (one-way ANOVA + Sheffe's post hoc test) versus control (*) and MA (†). αMpT (1 mM) and L-DOPA (500 μμ) were applied 24 h prior to MA. Intracellular DA levels were 1.5 ± 0.3, 0.42 ± 0.2, 2.1 ± 1.0 and 2.3 ± 1.0 nmol/mg protein in PC12 cells treated with buffer, αMpT, L-DOPA, and αMpT + L-DOPA, respectively.

Table 2 *S*-Apomorphine (*S*-APO) dose-dependently suppresses MA-induced inclusions in PC12 cells and enhances the protective effects of αMpT

Treatment	% MA-induced whorls/cell
Control	4 ± 1.1
S-APO 0.1 μM	3.4 ± 0.6
S-APO 1 μM	5.6 ± 1.0
S-APO 10 μM	12 ± 1.8
αMpT 1 mM	6.2 ± 1.2
MA 1 μM	100 ± 8.5*
S-APO 0.1 μM + MA 1 μM	96.7 ± 15.5*
S-APO 1 μM + MA 1 μM	52.3 ± 10.7†
S-APO 10 μM + MA 1 μM	7.8 ± 3.1†
αMpT 1 mM + MA 1 μM	48.5 ± 8.2†
S-APO 0.1 μM + αMpT 1 mM + MA 1 μM	50.6 ± 12.4†
S-APO 1 μM + αMpT 1 mM + MA 1 μM	8.5 ± 2.6‡

Values are mean ± SEM of 10–12 determinations. $p < 0.05$ (one-way ANOVA + Sheffe's post hoc test) versus control (*), MA (†), and αMpT + MA (‡). αMpT was applied 24 h, while *S*-APO was administered 2 h prior to MA to the PC12 cell culture.

fully prevented dose-dependently formation of methamphetamine-induced inclusions (Table 2). Moreover, when combining αMpT with a dose of *S*-apomorphine (1 μM) providing partial protection we achieved again full prevention of MA-induced inclusions.

Discussion

Our findings indicate that MA, one of the most harmful drugs of abuse, induces neuronal inclusions in the substantia nigra and corpus striatum but not in the frontal cortex of mice. These inclusions appear as membranous whorls, similar to

the inclusions found in cultured midbrain neurones treated with MA (Cubells *et al*. 1994; Larsen *et al*. 2002). By their morphology, MA inclusions can be identified as autophagic bodies rather than Lewy bodies, because Lewy bodies are not membrane-delimited. However, MA-induced inclusions showed some of the typical features of the inclusions found in chronic neurodegenerative disorders and modify their fine ultrastructure time-dependently. For example, MA inclusions found in the substantia nigra were immunopositive for α-synuclein, parkin and other proteins related to the ubiquitin/proteasome pathway, while maturation of the same inclusions in PC12 cells leads to the loss of the external membrane. Nigral inclusions are exclusively localized in the cytoplasm, similarly to Lewy bodies found in the substantia nigra or locus coeruleus of the PD brain. In contrast, striatal MA-induced inclusions are present in the cell nucleus and do not contain α-synuclein, as commonly observed in the PD brain (Forno 1996; Spillantini *et al*. 1997; Chung *et al*. 2001a, 2001b). It is noteworthy that inclusions are produced with doses of MA that induce a remarkable degeneration of nigro-striatal dopaminergic fibres as described here and previously reported in a dose–response study (Fornai *et al*. 1999). Our findings suggest that MA inclusions accompany MA neurotoxicity but are not necessarily related to neurodegeneration, it being possible that they might also reflect a defensive mechanism aimed at clearing harmful proteins. This point is particularly crucial since most reports indicate that nigral neurones do not die following administration of these doses of MA and we failed to observe nigral cell loss following a dosage of MA, which produces neuronal inclusions. Similarly, inclusions found in striatal neurones occur in the absence of cell loss. Finally, in PC12 cells the dose of MA which produces the highest number of inclusions (1 μM) does not produce cell death, while increasing the dose (up to 100 μM) leads to massive damage of the cell culture which does not allow to visualize cell inclusions. Therefore, it cannot be ruled out that inclusion formation might represent a defence mechanism, which is recruited when higher levels of oxidized, misfolded proteins occur in the cell. In fact, even when we evaluated PC12 cell cultures, we found a great number of cell inclusions in the absence of cell death. In this way, MA-induced inclusions might be transient and even beneficial for the cell. Therefore, inclusion formation might be a reactive phenomenon, which is recruited when a high, though tolerable, amount of oxidative stress is generated. In this case, the cell maintains its viability and it is capable of developing intracellular bodies. Interestingly, the onset of these inclusions can be prevented by the antioxidant, iron-chelating agent *S*-apomorphine. The potential importance of this process in the pathophysiology of parkinsonism induced by amphetamine derivatives (including MDMA or 'ecstasy') prompted us to further examine the nature of MA inclusions using cultured PC12 cells as a simple and homogenous cellular model. In these cells, MA-induced membranous whorls are found exclusively in the cytoplasm and contain α-synuclein, thus resembling the inclusions found in mouse nigral neurones. The use of these cultures allowed us to examine the formation of MA inclusions as a function of time. Inclusions were initially characterized by having multiple layers of membranes, which then fused, surrounding an electron-dense core. Therefore, when examining MA-induced inclusions at 1 week, they could no longer be considered as multilamellar bodies, featuring as non-membrane delimited inclusions with an electron-dense core. The dose of MA we selected for most *in vitro* studies (1 μM) produces a high amount of whorls in the absence of cell loss with only a slight decrease in DA stores. When raising the dose of MA up to 100 μM massive cell loss occurred with no evidence of inclusions. In this way, exposing PC12 cells to MA (1 μM) for 7 days allowed us to obtain a high amount of intracellular inclusions in the absence of significant toxicity (i.e. cell loss). Interestingly, DA has been implicated in the pathophysiology of Lewy bodies (Conway *et al*. 2001; Sulzer 2001), thus providing a further linkage between MA-induced inclusions and Lewy bodies. As nigral neurones and PC12 cells have in common the ability to synthesize DA, we wondered whether the availability of intracellular DA was a prerequisite for the formation of MA inclusions. In fact, it has been demonstrated that autophagic inclusions induced by MA *in vitro* are related to endogenous DA (Larsen *et al*. 2002). In particular, methamphetamine is known to produce high amounts of cytosolic DA which, in turn, might undergo oxidation to DA-quinone, both following an enzyme-dependent mechanism or via a concomitant reduction of iron(III) ions (Sulzer and Zecca 2000). This latter mechanism is responsible for the formation of autophagic granules and, again, it is highly reminiscent of what occurs in Parkinson's disease. The distribution of iron in Parkinson's disease has been investigated extensively by Youdim's group (Riederer *et al*. 1992). In fact, in the substantia nigra of parkinsonian brains, intraneuronal electron-dense neuromelanin granules contain iron(III) ions (Jellinger *et al*. 1992). Similarly, at an experimental level a mutual interaction between DA and iron(III) ions has been demonstrated and it might play a role in the progression of nigrostriatal degeneration (Linert *et al*. 1996). This is also in line with our present findings showing that the iron-chelating agent *S*-apomorphine dose-dependently prevents inclusion formation in PC12 cells. Similarly, inclusions were suppressed in PC12 cells following a combined administration of a low dose (1 μM) of *S*-apomorphine with the TH inhibitor, αMpT. In fact, blocking DA synthesis with αMpT partially prevents inclusion formation unless DA synthesis was restored by the addition of L-DOPA. L-DOPA also enhanced the formation of inclusions in the absence of αMpT or MA. This adds a new element to the long debate of whether L-DOPA treatment is toxic for surviving nigral neurones in parkinsonian patients (Blin *et al*. 1988). The

ability to prevent or enhance formation of inclusions by suppressing or augmenting, respectively, DA synthesis should not be regarded as a final proof for a direct role of DA in inducing neuronal inclusions. In fact, one might hypothesize that inclusions depend on other metabolic systems indirectly related to DA synthesis, these pathways might also be altered by various pharmacological treatments aimed at altering DA synthesis. When administering to PC12 cells the antioxidant iron-chelating agent *S*-apomorphine we observed again a significant suppression of inclusions. *S*-Apomorphine, at these doses, does not interact with DA receptors but it has been shown to protect PC12 cells and striatal DA terminals against oxidative stress (Gassen *et al*. 1998; Grunblatt *et al*. 2001). In line with this knowledge, in a previous study, we demonstrated that apomorphine was able to prevent methamphetamine toxicity *in vivo* with a mechanism which is independent of the stimulation of DA receptors (Fornai *et al*. 2001). Therefore, reducing DA synthesis is not the sole mechanism, which might attenuate inclusion formation. Indeed, occurrence of endogenous DA might contribute, but not be solely responsible for the generation of MA-induced oxidative stress; in this way, adding the antioxidant *S*-apomorphine provides a full prevention of inclusion formation, which is achieved only partially by inhibiting DA synthesis. DA might also be implicated in the development of inclusions observed in medium-sized striatal spiny neurones, which do not synthesize catecholamines. However, one should take into account that striatal neurones develop MA-induced neuronal inclusions but do not synthesize DA and are exposed only transiently to extracellular DA peaks; this is quite different when compared with nigral and PC12 cells which constantly synthesize DA. Thus, in considering striatal neuronal inclusions as a consequence of increased DA activity, one should hypothesize that DA receptors placed on striatal neurones might be crucial in transducing the metabolic signalling which triggers formation of inclusions within striatal cells. Alternatively, one should hypothesize that oxidative species formed within DA terminals diffuse to striatal GABA neurones. In this particular case, it could be the DA released in response to MA that, through the formation of radical oxygen species, impairs the ubiquitin-proteasome pathway in postsynaptic striatal neurones (Jakel and Maragos 2000). In this latter case, one should consider that the pathogenesis of methamphetamine-induced striatal inclusions is different to those observed in catecholamine containing cells, as the effects of DA would can be mediated either by signals transduced by DA receptors or by diffusion within striatal neurones of DA-derived oxidative species. To further explore this point we are investigating the effects of different doses of DA either alone or in combination with DA antagonists in cultured striatal neurones. Conversely, it would be worth trying MA in PC12 cell lines overexpressing DAT to verify whether MA produces stronger effects.

In summary, in the present paper we demonstrate that the widely abused amphetamine derivative methamphetamine produces neuronal inclusions *in vivo* within nigral and striatal, but not in fronto-cortical neurones, and we could not find inclusions in glial cells in any brain area under examination, even using a powerful analytical approach based on electron microscopy. These subcellular effects occur in the absence of cell loss as demonstrated both *in vivo* and *in vitro* in PC12 cells. In this latter experimental setting, we could demonstrate that inclusion formation occurs for doses of MA much lower (1 μM) than those required to produce massive cell death (100 μM). Long-term (7 days) exposure to this low (1 μM) dose of MA produces a massive filling of inclusions within each single PC12 cell under examination. This long-term exposure also allows us to appreciate the morphological maturation of the inclusions, which lose their external membranes resembling non-membrane limited neuronal inclusions, which occur in neurodegenerative disorders. Again, immuocytochemistry of these inclusions reveals a striking analogy with those occurring in neurodegeneration. Whether or not inclusions containing α-synuclein were found only in nigral neurones and PC12 cells because these cells share the ability to synthesize catecholamines is an additional intriguing question that warrants further investigation. Dopamine synthesis might play a role in promoting inclusion formation either directly or via alternative metabolic pathways. The role of iron(III), which has been demonstrated to play a role in the formation of DA-related oxidative damage and inclusions as well as other oxidant species, seems to contribute substantially to the genesis of MA-induced neuronal bodies. Administration of the iron chelator, antioxidant agent *S*-apomorphine prevents these inclusions.

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