Amphetamines induce apoptosis and regulation of bcl-x splice variants in neocortical neurons

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abstract **Amphetamine analogs have emerged as popular recreational drugs of abuse. The number of reports of these substances producing severe acute toxicity and death is increasing. In 'Ecstasy' -associated deaths, focal necrosis in the liver and individual myocytic necrosis has been reported. Furthermore, serotonergic and dopaminergic neuronal cell damage has been observed in experimental amphetamine intoxication in laboratory animals. Here we demonstrate that subchronic exposure to D-amphetamine, methamphetamine, methylenedioxyamphetamine, and methylenedioxymethamphetamine ('Ecstasy*) results in significant neurotoxicity in rat neocortical neurons in vitro. This neuronal cell death is accompanied by endonucleosomal DNA cleavage and differential expression of anti- and proapoptotic bd-$x_{L/s}$ splice variants. In addition, we observed pronounced induction of cell stress-associated transcription factor c-jun and translation initiation inhibitor p97 after amphetamine treatment. These data support that the neurotoxic effects of different amphetamines are extended to rat neocortical neurons and that apoptotic pathways are involved in amphetamine-induced neurotoxicity.—Stumm, G., Schlegel, J., Schafer, T., Wiirz, C., Mennel, H. D., Krieg, J.-C., Vedder, H. Amphetamines induce apoptosis and regulation of bd-x splice variants in neocortical neurons. FASEB J. 13, 1065-1072 (1999)**

Key Words: drug abuse • neurotoxicity • bcl gene family

Amphetamines and analogs known as 'Speed', 'Ice', 'Eve', or 'Ecstasy' (methylenedioxymethamphetamine, or MDMA)[2] are misused as psychostimulants and have become popular recreational drugs of abuse over the last decade. Users assume these drugs are safe because they believe that the drugs to not produce physical dependence and damage. However, a growing number of deaths after MDMA intoxication have been reported recently (1). Main clinical toxic features of amphetamines such as hyperthermia, circulation, and hepatic failure correlate with pathomorphological demonstration of acute myocyte and hepatocyte necrosis. Amphetamines have also been associated with teratogenesis and increased fetal and infant death rates related to maternal drug abuse comparable to the effects of cocaine (2, 3).

In addition, 'Ecstasy' and other amphetamine-derived drugs induce hallucinations as well as long-term neuropsychiatric alterations such as panic disorders, psychosis, and affective disorders (4-6). Very recently, quantitative positron emission tomographic evidence (PET) studies provided evidence of a decrease in brain 5-HT transporters in human MDMA users, strongly suggesting toxic effects of 'Ecstasy' in human serotonergic neurons (7). Indeed, for several years experimental data had demonstrated neurotoxicity to neurons of laboratory animals and non-human primates (8-11). So far, reports of the neurotoxic effects of amphetamines are focused to serotonergic and dopaminergic neurons, which are located mainly in midbrain structures. However, structural neuronal alterations have also been detected in the striatum and prefrontal cortex of amphetamine-treated rats, indicating a more general toxic process (12, 13). The underlying mechanisms of action in amphetamine neurotoxicity are still unknown, although there is some evidence for hydroxyl radical formation and for activation of apoptotic pathways (14-18).

The present study aimed to determine whether amphetamines deleteriously affect cortical neurons and, if so, which cell death-associated pathways are involved in the neurotoxic process.

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[2] Abbreviations: DA, D-amphetamine; DMEM, Dulbecco's modified Eagle's medium; MA, methamphetamine; MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine ('Ecstasy'); MEM, minimum essential medium; MTT, 3-(4,5-dimethyl-tetrazol-2-yl)-2,5-diphenyl-tetrazolium bromide; RT-PCR, reverse transcription polymerase chain reaction; PET, positron emission tomographic evidence.

MATERIALS AND METHODS

Cell culture

Primary cultures of fetal neurons were prepared as described previously (19). In brief, pregnant Wistar rats were killed after halothane narcosis by cervical dislocation and fetuses (E 18) were removed after midline incision. Neocortical regions were microdissected under sterile conditions and kept on ice in preparation medium (Dulbecco's modified Eagle's medium (DMEM)/25 mM HEPES). After mechanical dissociation of tissue, cells were centrifuged, resuspended in MEM (minimal essential medium) supplemented with antibiotics (antibiotic/antimycotic solution: 10,000 U penicillin, 10,000 pg streptomycin, 25 pg amphotericin B/ml in saline), 1 mM pyruvate, and 10% heat-inactivated fetal calf serum. They were then plated at a density of 1.75 X 10^5 cells/well in poly-D-lysine-coated 24-well dishes. After 24 h, the medium was replaced by a serum-free medium (START V). Cells were maintained at 37°C and 5% CO_2 at a humidity of 95-100% and used for experiments starting at day 11 of cultivation in vitro. Cell culture media and supplements were purchased from Seromed (Berlin, Germany), poly-D-lysine from Sigma (Deisenhofen, Germany), and cell culture dishes from Nunc (Wiesbaden, Germany).

Treatment with amphetamines

Cortical neurons were treated with 125, 250, 500, 750, and 1000 pM of D-amphetamine (DA), methamphetamine (MA), methylenedioxyamphetamine (MDA), and MDMA for 1, 24, and 96 h, respectively. All amphetamines were supplied by Sigma (München, Germany); stock solutions were prepared in phosphate-buffered saline and further diluted in medium under sterile conditions.

Quantification of cell viability

Cellular morphology was photographically documented by phase contrast microscopy after 1, 24, and 96 h of amphetamine treatment. In addition, cell viability after 96 h of exposure to amphetamines was quantified by a modified MTT [3-(4,5-dimethyl-tetrazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay (EZ4 U, Biozol GmbH, Eching, Germany). After 3.5 h of incubation at the end of the treatment period, absorption was measured at 490 nm in a microplate reader (Dynatech, Denkendorf, Germany). A 620 nm reference filter was used to correct for nonspecific background values. Data of the MTT assay represent results of four independent experiments. Differences between treatments were evaluated using Kruskal-Wallis one-way analysis of variance on ranks.

Analysis of DNA fragmentation

Isolation of genomic DNA and agarose gel electrophoresis

Genomic DNA was extracted from cortical neurons treated with 500 pM each of DA and MA for 96 h, as follows. Cells were washed in phosphate-buffered saline and rinsed in homogenization buffer containing 10 mM TrisCl (pH 7), 10 mM EDTA, and 0.6% sodium dodecyl sulfate. After 30 min of incubation with 10 pg/ml RNase A at 56°C, NaCl was added to an end concentration of 1 M and the mixture was incubated for an additional 2 h at 4°C. Protein precipitations were removed by 20 min of centrifugation at 4°C and 20,000 g, the supernatant was extracted first with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). Precipitation of DNA was performed with absolute ethanol overnight at —20°C and centrifugation at 20,000 gfor 20 min. After photometric quantification, 5 pg of each DNA sample were run on 1.6% agarose gel and visualized with ethidium bromide staining.

In situ biotinylation of DNA strand breaks and immunodetection

DNA fragmentation was determined in situ after 96 h of treatment with 500 pM of amphetamines and in control cells. For this purpose, we used the FragEL Klenow Kit from Calbiochem-Novabiochem (Bad Soden, Germany) according to the manufacturer's protocol. In brief, DNA strand breaks within the nuclei were labeled by biotinylated dNTPs and Klenow polymerase, and detected with a peroxidase-coupled secondary antibody. Visualization was performed by 3'-diaminobenzidine tetrahydrochloride.

RNA extraction and detection of gene products by reverse transcription-polymerase chain reaction (RT-PCR)

Total mRNA was extracted from cultured and untreated cells with the RNeasy total RNA isolation kit (Quiagen, Hilden, Germany). cDNA first-strand synthesis was performed, incubating 250 ng RNA of control and treated cells for 90 min at 37°C in a 20 pl standard RT reaction mix containing random hexamers (6.25 A_{260} units/ml), IX RT reaction buffer, 10 mM DTT, 62.5 pM dNTPs, and 100 U MMLV reverse transcriptase (Life Technologies, Eggenstein, Germany). First-strand cDNA (2.5 pl) was used as template for subsequent PCR, applying primers (MWG, Ebersberg, Germany) for

3-actin: forward: 5'TGGAGAAGAGCTATGAGCTGCCTG3';
reverse: 5'GTGCCACCAGACAGCACTGTGTTG3';
bax: forward 5'CCAAGAAGCTGAGCGAGTGTCTC3';
reverse: 5'AGTTGCCATCAGCAAACATGTCA3';
bcl-2: forward: 5'CACAAT CTCCCCCAGTTCACC3';
reverse: 5'CACAATCCTCCCCCAGTTCACC3';
bcl-x: forward: 5'GGAGAGCGTTCAGTGATC3';
reverse: 5'CAATGGTGGCTGAAGAGA3';
p97: forward: 5'CGTCAAAGGAAGAACTGCT3';
reverse: 5'AGGGGGATGTCAACCT C3';
c-fos: forward: 5'GGTGAAGGCCTCCTCAGACT3';
reverse: 5'CTGGCCGTCTCCAGTGCCAA3'; and
c-jun: forward: 5'TGAAGTGACCGACTGTTCTATG;
reverse: 5'CCAT TGCTGGACTGGATG3'.

PCR mix contained 1X PCR reaction buffer, 1.5 mM $MgCl_2$, 20 pM dNTPs, 100 nM primer, and 0.5 U Taq polymerase (Quiagen, Hilden, Germany). PCR products were separated by electrophoresis in 5% nondenaturating polyacrylamide gels and visualized by silver staining as described previously (20). Semi-quantitative analysis was assessed by using the NIH Image analysis program on a Macintosh Power PC.

RESULTS

Neurotoxicity of amphetamine analogs

Microscopic evaluation and photodocumentation of the amphetamine effects on cortical neurons revealed no morphological differences between untreated controls and amphetamine exposed cells after the first hour. The first neuritic cell damage became visible after 24 h of treatment. The neurotoxic effect was obvious within 96 h of incubation

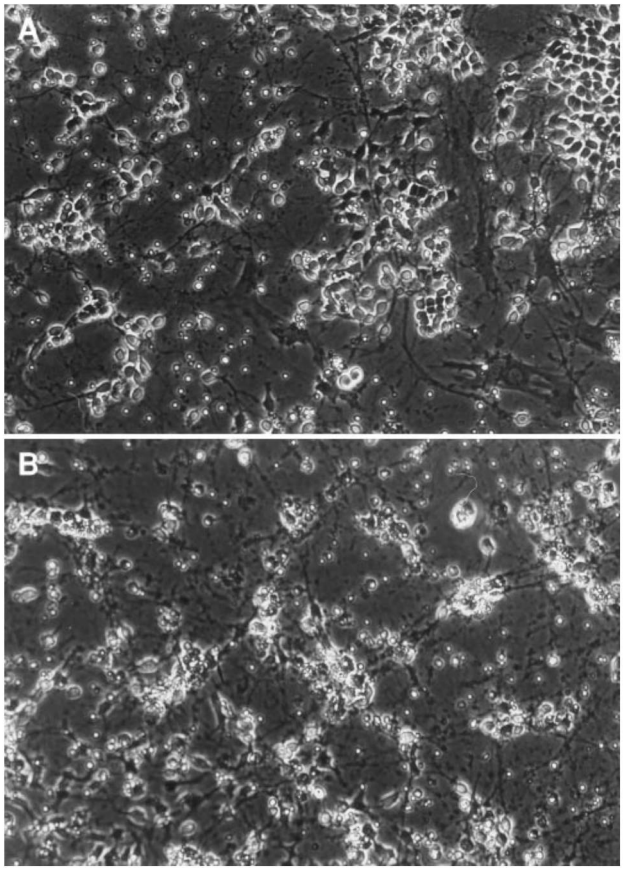

Figure 1. Primary rat cortical neurons are cultured in vitro for 14 days without amphetamine treatment (A). 96 h exposure to 500 pM D-amphetamine (day 11 to 14 of in vitro culturing) leads to neuritic damage and loss of viable neurons (B).

with amphetamines, as shown by destroyed neuronal dendrites and quantitative reduction of viable cells **(Fig.** 1).

This morphological effect of amphetamine treatment was underscored by quantification of neurotoxicity by using the MTT cell viability test. After an incubation period of 96 h, we observed dose-dependent toxic effects for all four compounds compared with untreated cells **(Fig.** 2A-D). Using a mean dosage of 500 pM of amphetamines, we found that all four amphetamine compounds induced a significant decrease in cell viability compared with control ($P<0.01$ in Kruskal-Wallis one-way analysis of variance on ranks). The nonmethylated derivatives DA and MDA showed a significantly higher neurotoxicity with 49.6% ± 16.8 (DA) and 43.3% ± 7.0 (MDA) cell viability than the methylated amphetamine compounds MA and MDMA, with neuronal survival rates of 74.8% ± 9.4 and 65.8% ± 11.4, respectively ($P<0.01$ in Kruskal Wallis) **(Fig. 3).**

DNA fragmentation

Genomic DNA extracted from untreated cortical neurons explanted and grown in vitro for 14 days showed a high proportion of high molecular weight DNA and only a distinct laddering of DNA due to endonucleosomal cleavage in the agarose gel electrophoresis. However, after 96 h treatment with 500 pM of DA and MA, the amount of apoptotically cleaved DNA dramatically increased and the typical DNA laddering phenomenon was present **(Fig. 4).**

Using the FragEL DNA fragmentation assay, we observed only 3-5% positive nuclei in the untreated control cells. However, after 96 h of 500 pM MA and DA treatment, prominent immunoreactivity and nuclear fragmentation were present in approximately 40% (MA)-70% (DA) of cells **(Fig. 5).**

Alteration of gene expression

Analysis of gene expression was performed after treatment with 500 pM of amphetamines DA, MDA, MA, and MDMA for 1, 24, and 96 h, respectively.

Using RT-PCR, we observed distinct differential expression patterns of the bcl-x_L (long) and bcl-x_S (short) isoforms during amphetamine treatment **(Fig. 6A).** Bcl-x_L was down-regulated by all four amphetamine compounds after 96 h of incubation. In contrast, the bcl-x_S isoform was induced by amphetamine treatment. Induction was observed as soon as 1 h after treatment with MA and MDMA and after incubation for 96 h with the nonmethylated compounds DA and MDA. Although up-regulation of the bcl-x_S splice variant was delayed in DA and MDA treatment, the intensity of induction after 96 h treatment was as prominent as for MA and MDMA. No significant transcriptional regulation was observed for bax and bcl-2 mRNA when compared with the expression of the housekeeping gene [3-actin (Fig- 6B).

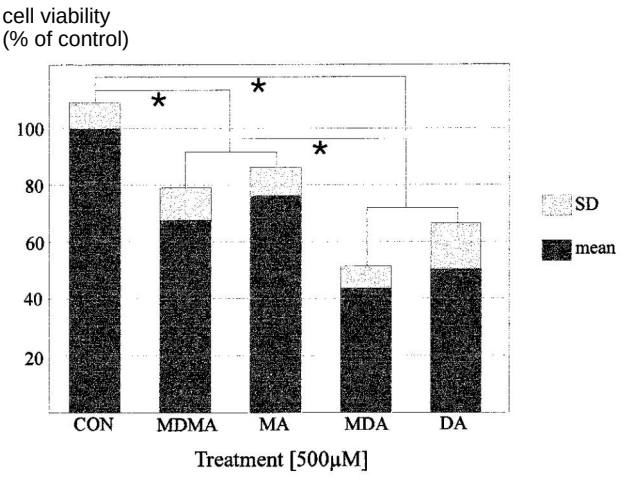

Figure 2. Dosage effects were tested for MDMA (A), MA (B), MDA (C), and DA (I)) in concentrations of 0, 125, 250, 500, 750, and 1000 pM for a treatment period of 96 h. MTT cell viability tests demonstrate dose-dependent toxic effects for all four amphetamine analogs.

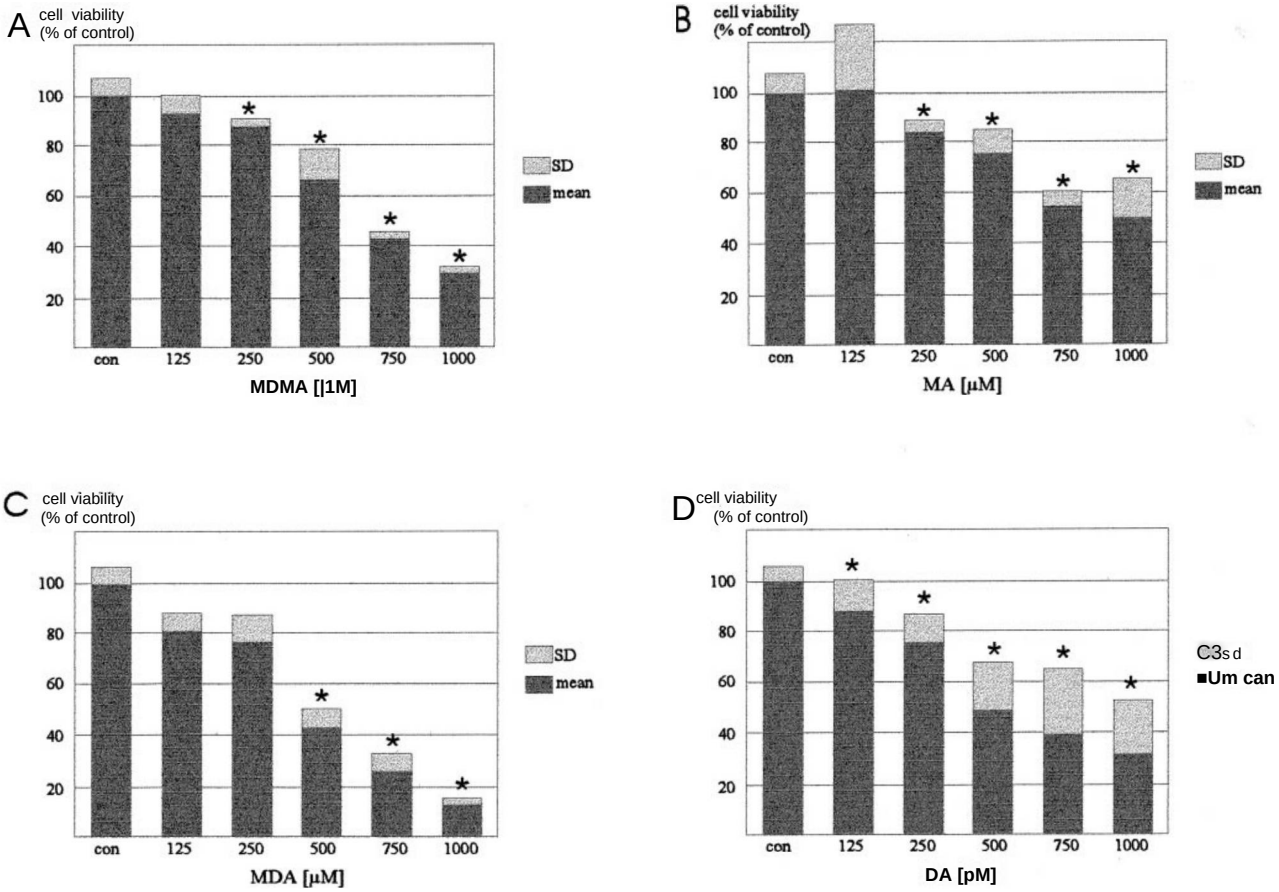

Figure 3. The comparison of MTT cell viability data of 500 pM treatment with MDMA, MA, MDA, and DA for 96 h exhibits a significant ($P<0.01$) neurotoxic effect of all four amphetamine compounds. DA and MDA treatment induces a significantly ($P<0.01$) higher neurotoxicity than the methylated MA and MDMA.

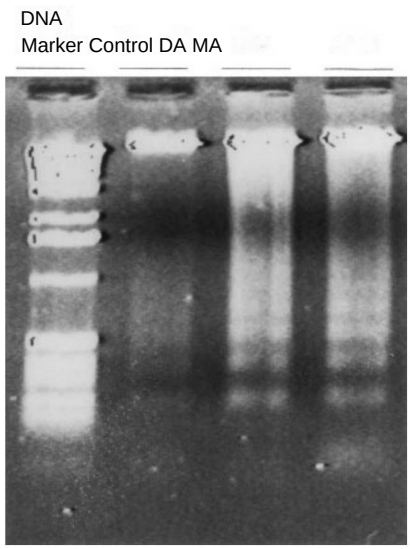

Figure 4. Genomic DNA isolated from untreated cortical neurons (control) and from cells exposed to MA or DA (500 pM each) for 96 h was separated in a 1.5% agarose gel electrophoresis. Control cells show only distinct DNA laddering, but MA and DA treatment dramatically enhances endonucleosomal DNA cleavage. The 1 kb ladder from Life Technologies was used as DNA size marker.

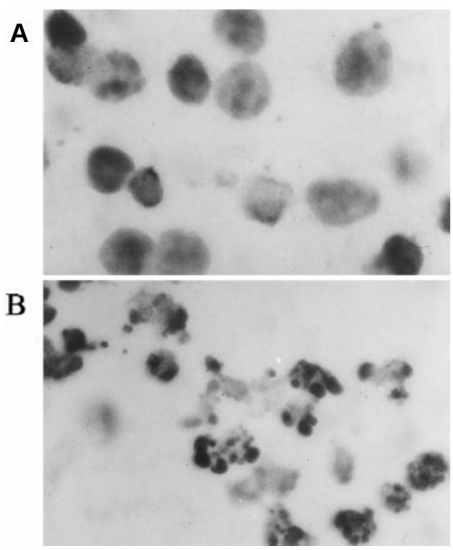

Figure 5. In situ detection of DNA fragmentation by the FragEL Kit is negative in the majority of untreated control cells (A), but positively labels more than 70% of cell nuclei after 96 h of 500 pM DA treatment (B). Besides the immunoreactivity, the typical apoptotic nuclear fragmentation is present.

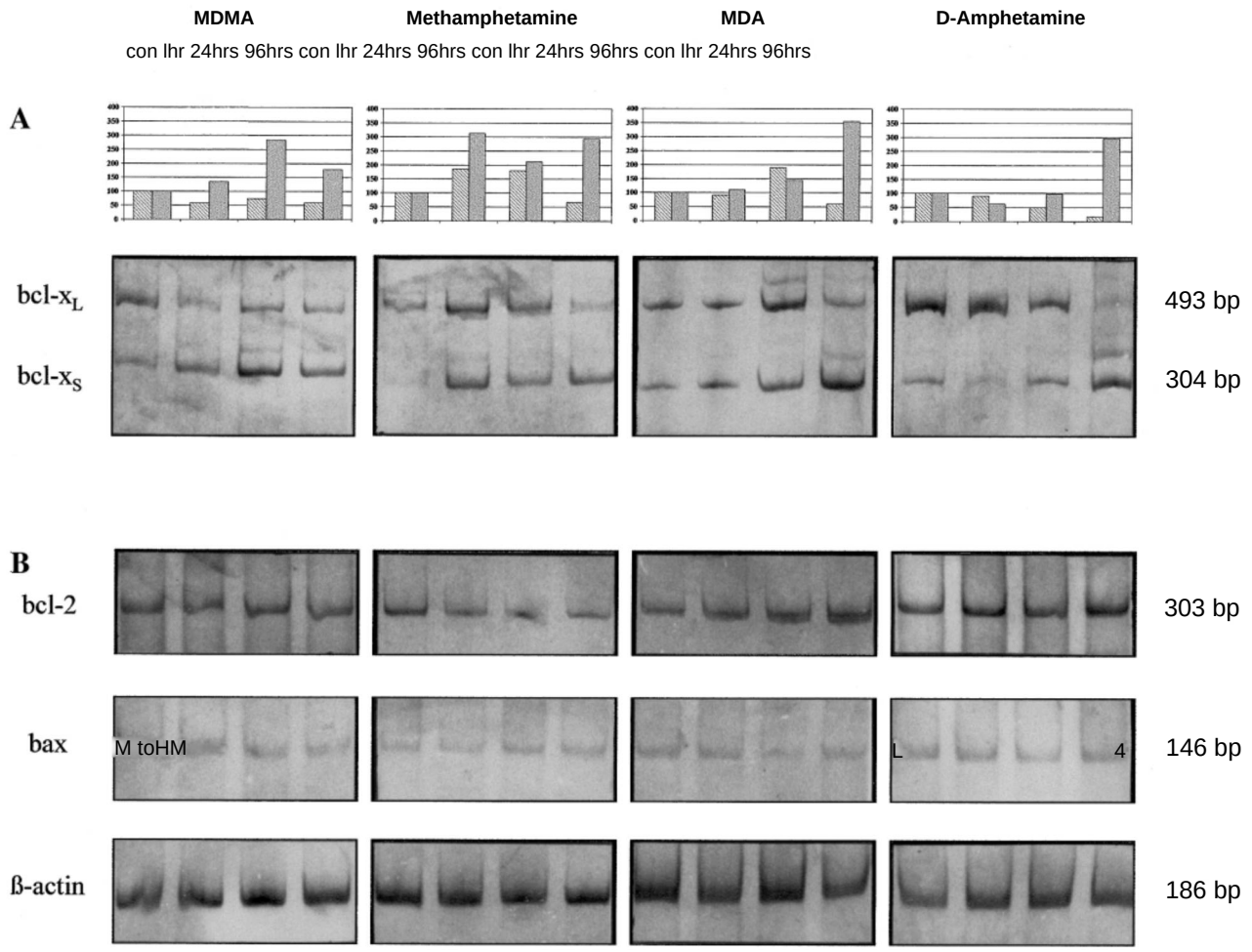

Figure 6. RT-PCR from RNA of control and amphetamine-treated cortical neurons shows differential regulation of the bcl-x splice variants with induction of the short bcl-x_s transcript and reduced transcription of the long bcl-x_L variant. (A). No regulation of bcl-2 or bax expression was observed in comparison to [i-actin (B).

The immediate early gene c-jun and the inhibitor of translation initiation p97 were up-regulated by both DA and MDA. Whereas a 2.5-fold induction of p97 mRNA was observed transiently after 1 h of drug treatment (Fig. 7.8), cjun showed a prolonged four-fold induction, with a peak at 24 h of treatment (Fig. 7/1). The methylated amphetamine analogs MA and MDMA did not significantly influence cjun or p97 transcription. No significant changes in c-fos mRNA levels were observed in any of the experiments (data not shown). RT-PCR results were confirmed by semi-quantitative analysis using the NIH Image analysis program (Fig. 6/1, Fig. 7/1, B).

DISCUSSION

The aim of the present study was to determine whether popular amphetamine drugs of abuse are able to direcdy damage rat cortical neurons in vitro. The role of cell stress- and apoptosis-associated pathways in amphetamine neurotoxicity was also investigated. We were able to demonstrate that subchronic exposure to either DA ('Speed'), MA ('Ice'), MDA, or MDMA ('Ecstasy') leads to significant neurotoxicity in rat neocortical neurons. Amphetamine-induced neuronal cell death was accompanied by endonucleosomal DNA cleavage and nuclear breakdown as well as differential expression of the anti- and proapoptotic bcl-$x_{L/s}$ splice variants, indicating an involvement of apoptotic pathways in amphetamine neurotoxicity.

Amphetamines are widely misused as psychostimulatory and hallucinatory agents. The analogs MDA and MDMA as main compounds of the designer drug 'Ecstasy' became appallingly prominent as recreational drugs of abuse during the last decade. Postmortem findings in accumulating human deaths associated with 'Ecstasy' intoxication resemble hepatocyte and myocyte necrosis as well as brain perivascular hemorrhagic and hypoxic changes (1). McCann and co-workers were able to demonstrate a

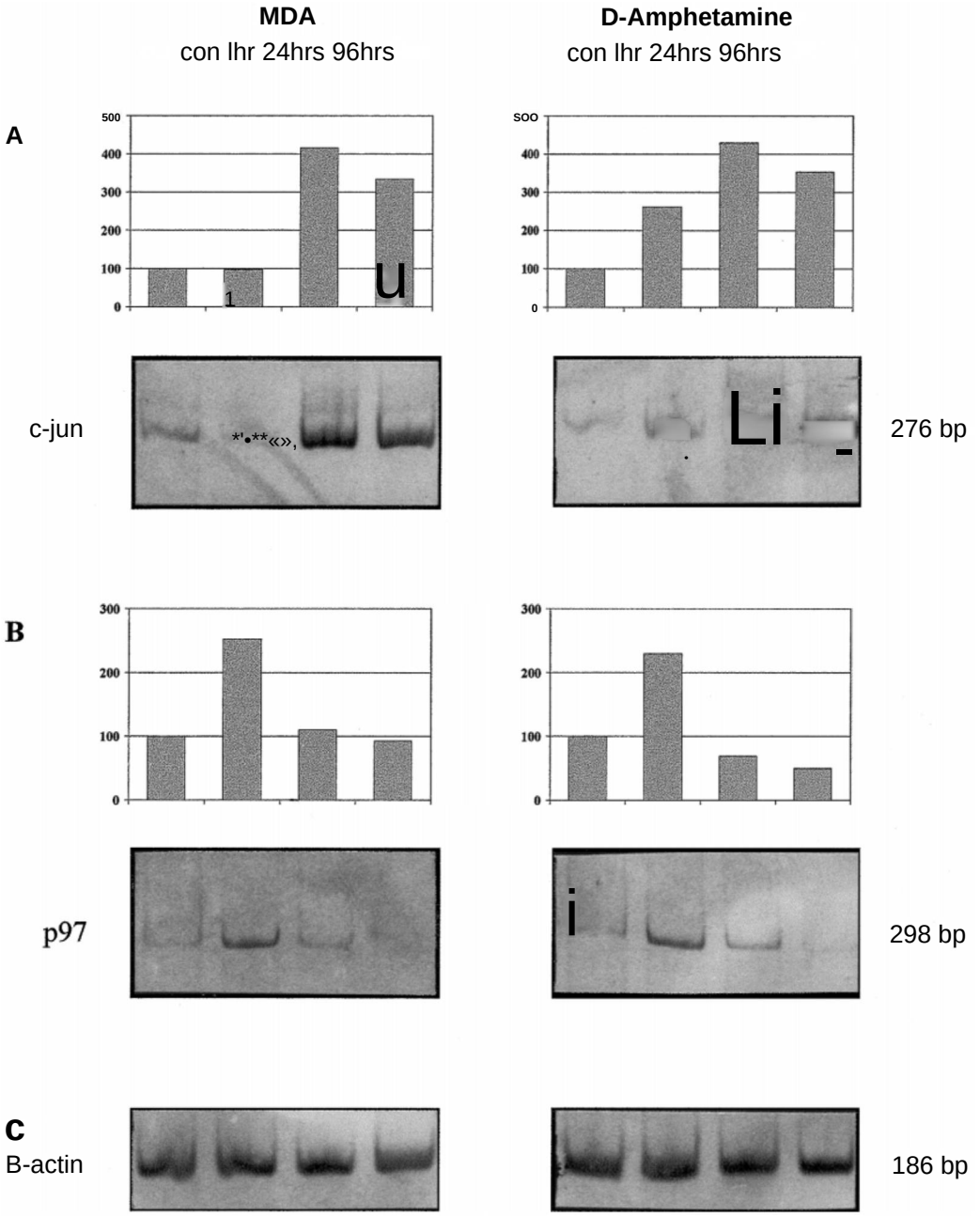

Figure 7. RT-PCR for cjun (A), p97 (B), and [3-actin (C) from RNA of control and amphetamine-treated cortical neurons demonstrates persistent induction of cjun, with a peak of fourfold expression compared to control after 24 h and transient 2.5-fold induction of p97 after 1 h treatment with MDA and DA

decrease in brain 5-HT transporters in abstinent human MDMA users by quantitative PET studies (7). Their data strongly suggest that 'Ecstasy' leads to long-lasting toxic effects in human serotonin neurons.

For a decade, animal experiments have provided evidence that certain amphetamine analogs have the potential to directiy damage central monoaminergic neurons. It has been shown in nonhuman primates and rodents that DA is toxic to dopaminergic neurons, MDMA to serotonergic neurons, and MA to both (8, 9). This neurotoxic effect and the amphetamine-induced behavioral syndrome are associated with a massive and rapid depletion of serotonin and dopamine storages by enhanced release and blocked reuptake of neurotransmitters (21). The underlying mechanism for neuronal cell damage is still unknown, but involvement of oxygen-based free radicals in the mediation of toxicity has been suggested by several authors (16-18, 22, 23). The attenuation of MA neurotoxicity in CuZn-superoxide dismutase transgenic mice and pretreatment with ascorbic acid supported the hypothesis that endogenous formation of 6-hydroxydopaminee and 5,7-dihydroxytryptamine might be responsible for the toxic effects. In this context, activation of apoptotic pathways by amphetamine intoxication has recendy been discussed. Cadet et al. (14, 24) demonstrated that MA neurotoxicity in rat neural cells is preventable by

overexpression of antiapoptotic bcl-2 protein and homozygous knockout of p53. Evidence for amphetamine-induced apoptosis has also come from Simantov and Tauber (15), who showed cell cycle arrest in G2M phase and DNA laddering in the human placental serotonergic cell line JAR after 48 h of MDMA and DA treatment.

Whereas earlier investigations have mainly been restricted to serotonergic and dopaminergic neurons, the present study focused on amphetamine neurotoxicity in rat cortical neurons. Induction of endonucleosomal DNA cleavage demonstrated by DNA laddering in the gel electrophoresis and in situ detection accompanies significant loss of cell viability in the primary cortical cell cultures. Furthermore, we were able to show differential expression of the bcl-$x_{L/s}$ gene during amphetamine treatment. The protective long-splice variant bcl-x_L, was down-regulated by amphetamines, whereas the contrary effect was observed for the proapoptotic bcl-x_s isoform, which was up-regulated during the progress of neuronal cell damage. In contrast, bax and bcl-2 expression were not affected by amphetamine treatment. These data are in accordance with the observation of Parasadanian et al. (25), who emphasized the role of bcl-x_L as an antiapoptotic regulator, especially for mature central neurons. They demonstrate that overexpression of bcl-x_L in transgenic mice prevents apoptosis of cortical and hippocampal neurons in a hypoxia-ischemia paradigm. However, a complete lack of bcl-x_L expression in knockout mice leads to extensive apoptotic neuronal cell death and lethality at embryonal day 13, underscoring the crucial role of bcl-x_L in the survival of postmitotic neurons (26). Therefore, the drug-dependent regulation of the bcl-$x_{L/s}$ variants observed during the progress of amphetamine neurotoxicity might be an important step in the induction of amphetamine-induced apoptosis of rat cortical neurons in vitro.

Regulation of pro- and antiapoptotic genes is well characterized in the hypoxia-ischemia paradigm and occurs simultaneously with changes in the expression pattern of the immediate early genes (27, 28). However, a direct functional connection between these gene families has not yet been established. Expression of eJun can be associated with both cell proliferation and cell death, depending on cofactors such as c-fos expression (29, 30). In ischemic neuronal cell damage, induction of eJun without coexpression of its AP-1 partner c-fos and inhibition of protein synthesis initiation are the most predictive markers for delayed neuronal death. In the present study we observed a prolonged induction of e Jun transcription after 24-96 h of treatment with the highly toxic DA and its ring substitute MDA. The methylated and less toxic MA and MDMA analogs, however, did not significandy alter the eJun RNA level. A c-fos induction was not present after either of the amphetamine treatments. These findings were underscored by the differential induction of p97 after amphetamine treatment. p97 inhibits initiation of protein biosynthesis as a competitive homologue of the initiation factor E4F (31). Similar to eJun, a transient up-regulation of p97 after 1 h treatment is also induced only by DA and MDA, but not by MA or MDMA.

In conclusion, our investigation demonstrates amphetamine-induced neurotoxicity in cortical neurons accompanied by apoptotic DNA fragmentation. Furthermore, we were able to associate amphetamine-induced differential regulation of the bcl-$x_{L/s}$ splice variants with neuronal cell death. Down-regulation of the protective bcl-x_L and simultaneous induction of the proapoptotic bcl-x_s variant accompany the neurotoxic effect. Expression of the immediate early transcription factor eJun and the translation initiation inhibitor p97 are restricted to the nonmethylated DA and MDA analogs and thereby to high neurotoxic potential. [D]

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