The abused drug MDMA (Ecstasy) induces programmed death of human serotonergic cells

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ABSTRACT **The widely abused amphetamine analog 3,4-methylenedioxymethamphetamine (MDMA, also called "ecstasy") induces hallucination and psychostimulation, as well as long-term neuropsychiatric behaviors such as panic and psychosis. In rodents and monkeys, MDMA is cytotoxic to serotonergic neurons, but this is less clear with humans. Herein, MDMA was cytotoxic to human serotonergic JAR cells; it altered the cell cycle, increased G2/M phase arrest, and induced DNA fragmentation in a cycloheximide-sensitive way. This apoptosis was not observed in nonserotonergic human NMB cells. The stereospecific effect of amphetamines in JAR cells, and the key role of NO and dopamine in MDMA-induced apoptosis were determined. The relevancy of MDMA-induced cell death to drug users is discussed.—Simantov, R., Tauber, M. The abused drug MDMA (Ecstasy) induces programmed death of human serotonergic cells. *FASEB J.* 11, 141–146 (1997)**

Key Words: apoptosis · drug abuse · transporter · psychosis · nitric oxide · neurodegeneration

ABUSED AMPHETAMINE ANALOGS, INCLUDING 3,4-methylenedioxymethamphetamine (MDMA, also called "ecstasy"),[2] are cytotoxic to rodent and monkey serotonergic neurons (1–6). The mechanisms underlying this neurotoxicity are associated with the cross talk between the drug and several neurotransmitter pathways; nevertheless, the details of these processes are yet unknown. MDMA induces the release of serotonin (7–9), most likely upon interaction with the recently cloned (10–12) serotonin transporter (SERT), and decreases serotonin levels in the brain (ref 13, and references therein). MDMA-induced neurotoxicity and serotonin release are both restrained by serotonin uptake inhibitors, but certain amphetamine derivatives that enhance serotonin release are not cytotoxic (14). Moreover, reports of human volunteers that consumed both MDMA and the selective serotonin uptake blocker fluoxetine (PROZAC) suggest that the reinforcing and neurotoxic effect of MDMA are separable (15). Other aminergic neurons, particularly dopamine, play a role in MDMA neurotoxicity; MDMA induces the release (and synthesis) of dopamine (16–19), certain amphetamines activate the biosynthesis of 6-hydroxydopamine (20), a neurotoxic dopamine derivative, and short-term depletion of the rat brain from dopamine reduces MDMA neurotoxicity (21).

Analyzing the MDMA mode of action in human cells is important in several aspects. First, the metabolism of amphetamines in primates (monkeys) is different from that in rodents (22). Second, the neurotoxic activity of MDMA in rodents was observed mainly at the level of neuronal processes, whereas in monkeys the cell bodies were also destroyed (1–6). This may be related to the finding that primates are more sensitive than rodents to the toxic activity of this drug (5, 6, 23–25). Rodents exhibit, overall, transient anatomical abnormalities upon treatment with the drug, whereas changes in monkeys are long lasting. Such long-term effects could be related to neuropsychiatric abnormal behavior, such as panic and psychosis (26–30), observed in MDMA drug users.

The aim of this study was, therefore, to study MDMA toxicity in human cells. The placental serotonergic cell line JAR, taking up serotonin (31, 32) and expressing the serotonin transporter SERT (12, 33, 34), was suitable for this aim. In addition to the importance of being human cells, JAR cells are advantageous on other model systems (8, 35) that contain only a small percentage of serotonergic cells. The use of JAR cells enabled us to shed light on intracellular pathways that participate in the process of MDMA-induced cell death. A human dopaminergic cell line (NMB) that expresses dopamine, but not serotonin transporters (36), was used throughout the study to verify the selectivity of the drug.

MATERIALS AND METHODS

Materials

Agarose, DNase-free RNase, ethidium bromide, sodium nitroprusside (NAP), propidium iodide, lactate dehydrogenase (LDH) kit, and 4,6-

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[2] Abbreviations: MDMA, 3,4-methylenedioxymethamphetamine; SERT, serotonin transporter; NAP, sodium nitroprusside; LDH, lactate dehydrogenase; L-NAME, N^G-nitro-L-arginine methyl ester; DMEM, Dulbecco modified Eagle medium; SNAP, S-nitroso-N-acetylpenicillamine; DAPI, 4,6-diamidino-2-phenylindole; FACS, flow cytometric analysis; NMB, a human dopaminergic cell line.

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diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, Mo.). N^G-nitro-L-arginine methyl ester (L-NAME), D-NAME, D-amphetamine, L-amphetamine, and S-nitroso-N-acetylpenicillamine (SNAP) were from RBI Co. MDMA was extracted from pellets kindly supplied by Drs. Y. Almog and S. Levy, Israel Police, Department of Forensic Identification, Jerusalem. All other chemicals were of analytical grade.

Cell cultures

The human choriocarcinoma placental JAR cells were purchased from the American Type Culture Collection (ATCC, Rockville, Md.) and cultured in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal calf serum, in a 10% CO_2 humidified incubator at 37°C. The human neuroblastoma NMB cells (37) were kindly provided by Drs. M. N. Goldstein and Y. Wollman, and adapted to DMEM with 10% Hyclone calf serum.

Cytotoxicity evaluation, DNA fragmentation, flow cytometric analysis, and LDH assay

Viability, morphological evaluation, and flow cytometric analysis (FACS) were determined 18–22 h after treatment with the indicated concentration of MDMA or amphetamines. Dopamine, serotonin, or L-DOPA were added in the indicated experiments 60 min before MDMA. Prior to photography, cells were fixed and stained on the culture plates by May-Grinwald/Giemsa method. For fluorescent staining with 4,6-diamidino-2-phenylindole (DAPI), cells were fixed with 3.7% formaldehyde, treated with 0.5% Triton X-100, stained with DAPI for 15 min, and mounted. For FACS analysis, cells were counted, labeled with propidium iodide, and analyzed (5000 cells/sample) on a Beckon Dickinson FACScan.

DNA fragmentation was determined as follows: Cells were washes with PBS, homogenized with 10 mM Tris-HCl buffer (pH 7.0) containing 10 mM EDTA and 0.6% SDS, treated with RNase (30 min at 37°C), and further treated with 1 M NaCl for 2 h at 4°C. After centrifugation at 14000 × *g*, the DNA (in the supernatant) was extracted first with phenol-chloroform-isoamyl alcohol, then with chloroform-isoamyl alcohol, and finally precipitated with absolute ethanol. Samples of 15–20 μg DNA were run on a 1.5% agarose gel. Ethidium bromide was used to mark the DNA bands.

The neurotoxic activity of MDMA or amphetamines was also determined by monitoring the release of the cytosolic enzyme LDH to the culture medium. Aliquots of 50 μl of the culture medium were used to test this enzyme, with the LDH assay kit of Sigma. Statistical analysis was conducted with the StatView program, and significance was determined with Student's *t* test and analysis of variance.

RESULTS

MDMA effect on viability, cell cycle, and DNA fragmentation in human cells

MDMA had dose- and time-dependent morphological effects on JAR cells; within 12 h the cells shrunk, the cell membrane lost its brightness and smoothness, the nucleus was smaller and darker, and cell fragments and debris appeared in the culture medium (**Fig. 1*A–D***). A dose-dependent effect on cell survival was apparent as well; e.g., treatment with 0.4–1.2 mM MDMA for 48 h decreased cell number by 14 ± 4–61 ± 9% (**Fig. 2*a***). This decrease in viability was accompanied with a dose-dependent increase in the amount of the cytosolic enzyme lactate dehydrogenase in the culture medium (Fig. 2*b*). The selectivity of MDMA cytotoxicity was further indicated with the serotonin transporter blocker imipramine, which at 1 μM inhibited significantly the effect MDMA (Fig. 2*c*). Lower concentrations of imipramine (0.1 and 0.01 μM) had a partial and dose-dependent effect (data not shown). Cocaine, having a higher affinity to dopamine transporters, had no such effect at 5 μM (Fig. 2*c*). The more selective dopamine transporter blocker GBR 12909 was tested at 1 and 5 μM, and was also inactive. Moreover, MDMA at 0.6 or 1.2 mM was not toxic to the dopaminergic human NMB cells (Fig. 2*d*), further indicating the selective effect of the drug.

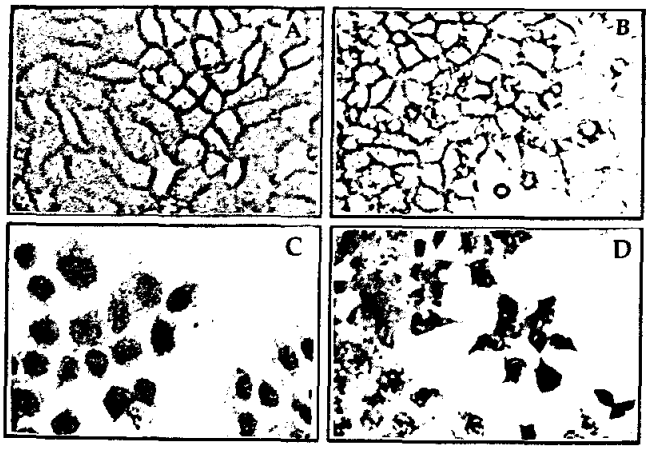

Figure 1. MDMA effect on the morphology of human serotonergic JAR cells. *A, C)* Control cells. *B, D)* Cells treated for 24 h with 0.6 mM MDMA. *A, B)* Phase-contrast photos of unstained cells. *C, D)* Giemsa-stained cells.

In an attempt to explain the mode of MDMA cytotoxicity, the DNA of untreated and MDMA-treated JAR or NMB cells was extracted and analyzed on agarose gel. **Figure 3*a*** (lanes 5–8) shows that in JAR cells, MDMA induced intranucleosomal DNA fragmentation, which appeared as a characteristic DNA ladder. Under the same conditions, the drug did not induce DNA fragmentation in NMB cells (Fig. 3*a*, lanes 1–4). A typical DNA fragmentation and apoptotic morphology in MDMA-treated JAR cells was also observed by fluorescent staining with 4,6-diamidino-2-phenylindole (DAPI) (Fig. 3*b*).

FACS indicated that MDMA altered the cell cycle profile of JAR cells and increased the percent of subdiploid DNA particles from 2.3 ± 0.4% in control cells to 19.5 ± 1.3% in treated cells (**Fig. 4*a, b***). At the same time, the percent of cells in the G1 phase was decreased from 62 ± 9% to 48 ± 11%, with a concomitant increase in the G2/M phase arrest (Fig. 4*a, b*). However, MDMA-treated NMB cells showed the same cell cycle profile as untreated NMB cells (Fig. 4*d, e*).

We then analyzed whether MDMA cytotoxicity in JAR cells depends on the activation of protein synthesis. **Table 1** shows that the inhibitor of protein synthesis cycloheximide blocked the cytotoxic effect of MDMA. Cycloheximide also blocked the alterations in the cell cycle profile induced by MDMA (Fig. 4*c*), as well as DNA fragmentation (data not shown).

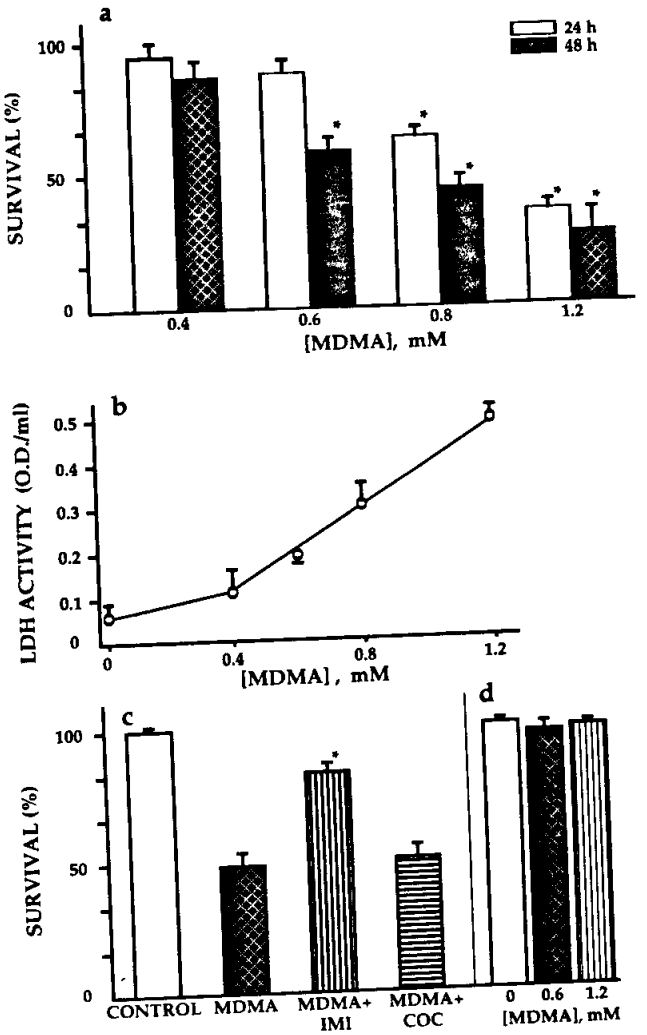

Figure 2. Cytotoxicity of MDMA in JAR cells, blockade by a serotonin uptake inhibitor, and cell selectivity. *a*) Cells were treated with different MDMA concentrations for 24 or 48 h, and cell survival was determined. Data are mean ± SD from experiments replicated three–five times. Asterisks (*) indicate a significant difference from control cells, $P < 0.01$. *b*) Lactate dehydrogenase (LDH) activity in the culture medium collected from JAR cells treated for 48 h with 0–1.2 mM MDMA. Data are mean ± SD of four–six replicates. *c*) Inhibition of MDMA toxicity in JAR cells with the serotonin uptake blocker imipramine. Cells were pretreated with imipramine (IMI) or cocaine (COC) 60 min before the application of 0.8 mM MDMA, and viability was determined 22 h later. Data are mean ± SD from experiments replicated four times. Asterisk indicates a significant difference from cells treated with MDMA, $P < 0.01$. *d*) No effect of two MDMA concentrations (48 h treatment) on the viability of human dopaminergic NMB cells.

An indication for the stereospecificity of MDMA effect was the finding that D-amphetamine was more cytotoxic than L-amphetamine (Table 1); this is in line with pharmacological studies of experimental animals (8, 38).

Role of nitric oxide and catecholamines

To identify intracellular pathways involved in MDMA cytotoxicity, analysis of the role of nitric oxide, an endogenous messenger that also has toxic activity, was performed. **Figure 5*a*** shows that the selective inhibitor of the enzyme nitric oxide synthase, N^G-nitro-L-arginine methyl ester (L-NAME), suppressed both MDMA and D-amphetamine cytotoxicity. Inhibition of MDMA toxicity by L-NAME was dose dependent, and L-NAME was more effective than its stereoisomer D-NAME (data not shown). Furthermore, the nitric oxide donor NAP decreased cell survival dose dependently (Fig. 5*b*). NAP also induced DNA fragmentation and ladder formation (Fig. 5*c*). SNAP, another nitric oxide donor, was also cytotoxic (Fig. 5*b*).

As discussed above, the involvement of catecholamine neurotransmitters in MDMA toxicity in vivo is still unknown, particularly in human cells. The cultured JAR cells were, therefore, useful in determining

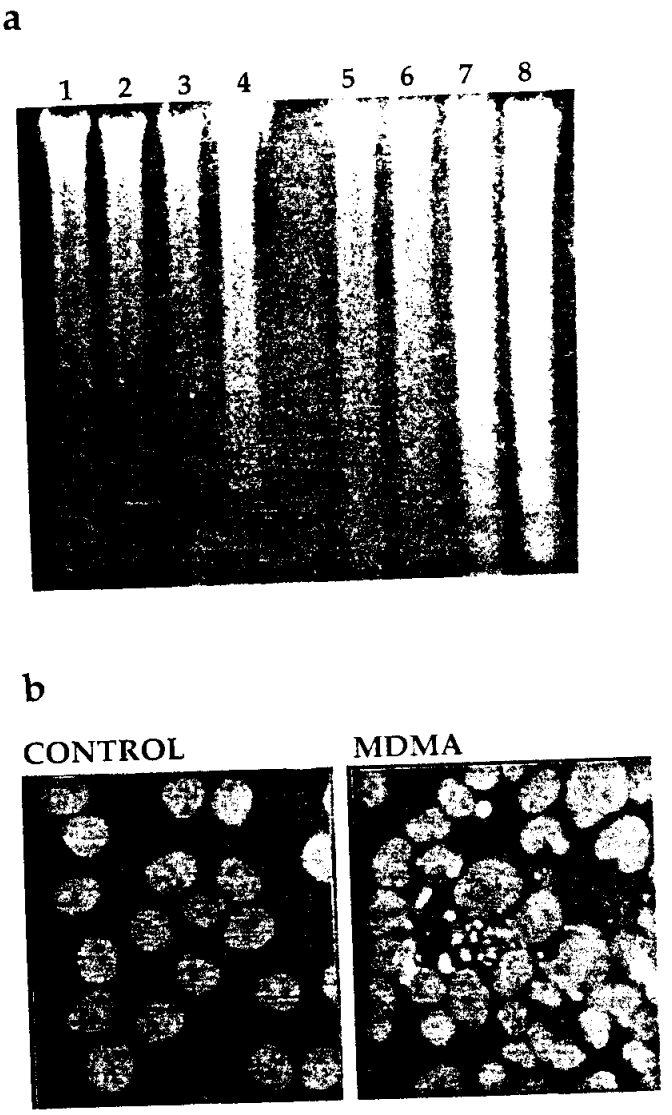

Figure 3. MDMA-induced DNA fragmentation. *a*) DNA was extracted from dopaminergic NMB (lanes 1–4) or serotonergic JAR (lanes 5–8) cells, untreated (lanes 1, 2, 5, and 6), or treated for 18 h with 1.2 mM MDMA (lanes 3, 4, 7, and 8). DNA was extracted and analyzed as described in Materials and Methods. *b*) DAPI staining of control or MDMA-treated (0.8 mM for 18 h) JAR cells.

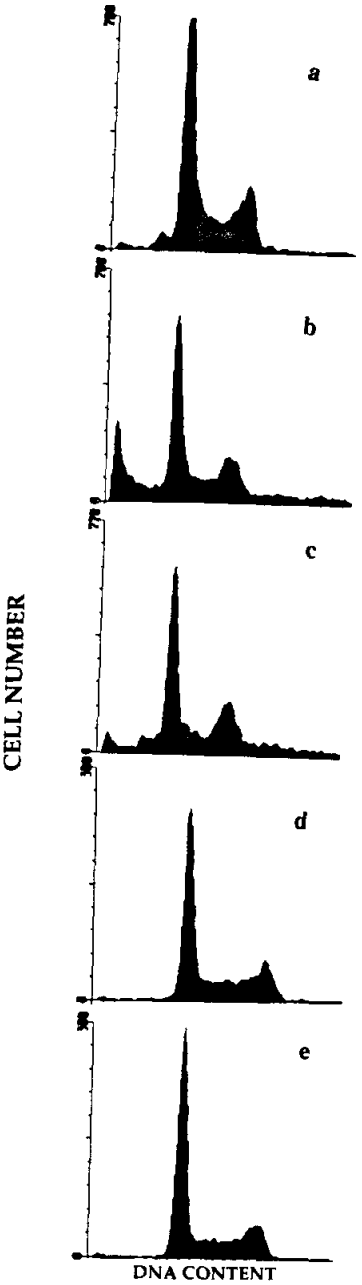

Figure 4. Flow cytometric analysis of JAR and NMB cells treated with MDMA. *a*) Control JAR cells. *b*) JAR cells treated with 0.6 mM MDMA for 18 h. *c*) JAR cells treated the same as panel *b*, but with 0.25 μM cycloheximide. *d*) Control NMB cells. *e*) NMB cells treated with 0.6 mM MDMA for 18 h. Cells were analyzed for the cell cycle as described in Materials and Methods. Data are from a representative experiment replicated three times. Cycloheximide by itself (at 0.25 μM) had no effect on the cell cycle of JAR cells (data not shown).

possible interaction, at the cellular level, between MDMA and catecholamines. Table 1 shows that dopamine, but not serotonin, increased MDMA toxicity. In line with the experiments described above, dopamine also increased the toxic activity of NAP (**Table 2**). Moreover, the dopamine precursor L-DOPA enhanced the toxic activity of both NAP (Table 2) and MDMA (data not shown).

DISCUSSION

MDMA, a widely abused recreational drug, has long-term neuropsychiatric effects (26–30). The cytotoxic effect of MDMA was extensively analyzed in rodent and monkeys, but not in humans (1–6). In the current study, a human serotonergic cell line was used to unravel certain intracellular pathways mediating the activity of this amphetamine analogue. MDMA induced intranucleosomal DNA fragmentation, altered the cell cycle profile, and increased the percentage of subdiploid DNA particles. Our findings suggest that the drug induces apoptotic processes that depend on protein synthesis.

The role of the serotonin transporter in MDMA cytotoxicity was approached in several ways: Imipramine, a serotonin transporter blocker, inhibited MDMA cytotoxicity in JAR cells, whereas the dopamine transporter inhibitor cocaine was inactive. Second, the drug was not toxic to human neuronal NMB cells, expressing dopamine but not serotonin transporter (36). The observation that D-amphetamine was more toxic than L-amphetamine to JAR cells is in line with the notion that MDMA effect is also stereospecific (8, 38). The study indicates, therefore, that the serotonin transporter plays a key role in MDMA cytotoxicity in human cells.

Regarding the mechanism of MDMA neurotoxicity in the brain, the role of the neurotransmitters serotonin and dopamine is unknown (see refs 13, 39 for review). The neuroanatomical complexity of dopaminergic and serotonergic pathways makes this issue a difficult one to resolve in vivo. Herein, it was shown that cultured JAR cells were more vulnerable to MDMA in the presence of dopamine,

TABLE 1. *Effect of cycloheximide (CHX), serotonin, and dopamine on MDMA cytotoxicity, and the stereospecific toxicity of amphetamines*[a]

Treatment	Concentration	Viability ($\times 10^3$/plate)
Part a		
None	—	325 ± 34
MDMA	0.6 mM	204 ± 47*
CHX	0.25 μM	315 ± 53
MDMA + CHX	0.6 mM + 0.25 μM	310 ± 49**
D-Amphetamine	0.5 mM	227 ± 30*
L-Amphetamine	0.5 mM	334 ± 47
Part b		
None	—	235 ± 15
MDMA	0.8 mM	133 ± 16*
Serotonin	0.1 mM	214 ± 17
MDMA + serotonin	0.8 mM + 0.1 mM	120 ± 13
Dopamine	0.1 mM	225 ± 24
MDMA + dopamine	0.8 mM + 0.1 mM	73 ± 7**

[a] Data of part a are mean ± SD from three or four experiments, each performed in duplicate. An analysis of variance have shown a significant effect, $F(5,22) = 55.4$ and $P < 0.01$. Single and double asterisks indicate significant difference from control or MDMA-treated cells, $P < 0.005$ and 0.01, respectively. Data of part b are mean ± SD from three experiments run in duplicate. An analysis of variance have shown a significant effect, $F(5,17) = 43.7$ and $P < 0.001$. Single and double asterisks indicate significant difference from control and MDMA-treated cells, $P < 0.001$ and 0.01, respectively.

whereas serotonin had no such effect. The immediate in vivo effects of MDMA, inhibition of the serotonin transporter and enhanced release of serotonin, increase the extracellular level of serotonin and the activation of pre- and postsynaptic serotonin receptors. MDMA also blocks monoamine oxidases (preferentially the A type) (40), which further decreases the rate of serotonin removal from the synapse. These fast changes in serotonin transmission induce, within several days, a depletion of serotonin (41, 42). Indeed, pretreatment of rats with the serotonin precursors tryptophan and 5-hydroxytryptophan attenuates MDMA neurotoxicity (42), indicating that serotonin depletion is a key element in the process of MDMA-induced cell death. As serotonin did not increase MDMA toxicity in JAR cells, it is apparent that this neurotransmitter does not enhance MDMA toxicity directly.

TABLE 2. *Effect of dopamine and L-DOPA on sodium nitroprusside (NAP) toxicity*[a]

Treatment	Concentration (mM)	Viability ($\times 10^3$/well)
None	—	157 ± 23
NAP	0.4	132 ± 17
Dopamine	0.25	140 ± 26
NAP + Dopamine	0.4 + 0.25	74 ± 14*
L-DOPA	0.5	150 ± 22
NAP + L-DOPA	0.4 + 0.5	86 ± 15*

[a] Data are mean ± SD from two or three experiments run in 24-well multiplates, assayed in duplicate. An analysis of variance have shown a significant effect, $F(5,15) = 27.7$ and $P < 0.01$. Asterisk indicates significant difference from NAP-treated cells, $P < 0.01$.

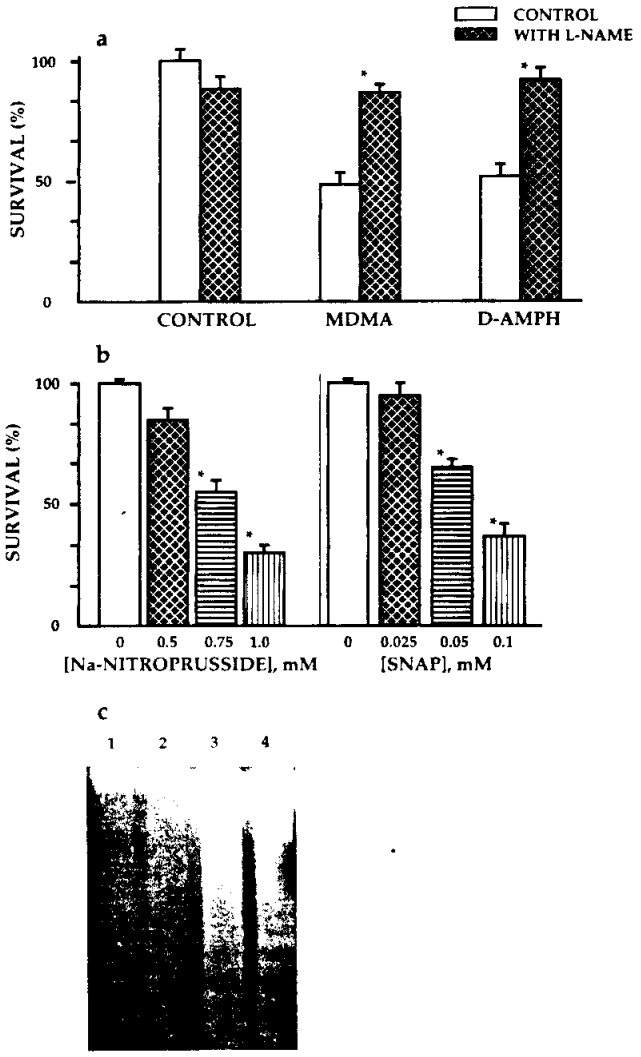

Figure 5. Inhibition of MDMA toxicity with the inhibitor of nitric oxide synthase L-NAME, and cytotoxicity of Na-nitroprusside (NAP) and S-Nitroso-N-acetylpenicillamine (SNAP). *a*) Survival of JAR cells treated for 48 h with 0.8 mM MDMA or 0.5 mM D-amphetamine, without or with 1 mM L-NAME. *b*) Effect of NAP and SNAP on cell survival. Data are mean ± SD from experiments replicated three times. Asterisks in panels *a* and *b* indicate significant difference from cells treated with only MDMA or from control cells, respectively, $P < 0.005$. *c*) Effect of NAP on DNA fragmentation. Lanes 1 and 2, control cells; lanes 3 and 4, cells treated with 1 mM NAP for 20 h.

With regard to dopamine, MDMA enhances dopamine synthesis and release in the striatum via the activation of 5-HT_2 receptors (16–19). Dopamine release was also observed in the nucleus accumbens, resulting from MDMA activation of dopaminergic neurons in the substantia nigra (42–44). Brodkin et al. (21) have shown that an acute dopamine depletion decreased the effect of MDMA. The enhanced toxicity of MDMA to JAR cells, in the presence of dopamine, suggests that this neurotransmitter (or its oxidized metabolites) work in concert with MDMA to induce cell death. We suggest, therefore, that dopamine interacts with MDMA at the cellular (metabolic) level, whereas serotonin activates MDMA neurotoxicity (in vivo) by enhancing dopamine release, via the activation of 5-HT_2 receptors.

Another aspect of this study is the role of NO in MDMA cytotoxicity; our results with JAR cells suggest that MDMA cytotoxicity is associated with, and possibly mediated by, the production of NO. It is well established that NO regulates the release (45) and uptake (46, 47) of both dopamine and serotonin. The enzyme responsible for NO synthesis, nitric oxide synthase, is calcium and calmodulin dependent (48), and these two messengers are involved in 5-HT_3 receptor activity (49). Azmitia and associates (35) have shown that MDMA is toxic to serotonergic neuronal cultures in a calcium-dependent way. Our observation that dopamine enhances the cytotoxic activity of both MDMA and the NO donor NAP supports the idea that MDMA toxicity to human cells is associated with NO metabolism. Indeed, recent experiments showed that MDMA regulates the activity of nitric oxide synthase (R. Simantov and M. Tauber, unpublished results). Unraveling the exact role of nitric oxide and dopamine in MDMA-induced programmed cell death will be useful for the development of approaches to block the neurotoxic activity of this abused drug. FJ

The studies in this laboratory were supported by grants from the Israel Antidrug Authority, Israel Ministry of Health, and the Leo & Julia Forchheimer Center for Molecular Genetics, the Mella and Leon Be-

rozivo Center for Neurosciences, and the Murray H. and Meyer Grodetsky Center for Research of Higher Brain Functions at the Weizmann Institute. The assistance of Dr. S. Porat in the DNA fragmentation assay is acknowledged.

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Received for publication June 28, 1996.
Accepted for publication November 20, 1996.