

Morphological Effects in the Mouse Myocardium After Methylenedioxymethamphetamine Administration Combined With Loud Noise Exposure

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ABSTRACT

Early toxicity occurring during or immediately after 3,4-methylenedioxymethamphetamine (MDMA, or “ecstasy”) administration has not been investigated in detail, although in humans it is responsible for marked side effects, and even death. Acute toxicity induced by MDMA produces rhabdomyolysis involving the myocardium (myocytolysis). Cardiac symptoms, such as tachycardia, hypertension, and arrhythmia, are present to a variable extent in humans abusing ecstasy. In most cases, this substance is abused in the presence of loud noise, which may affect the myocardium. Despite the frequency of the concomitant exposure to ecstasy and loud noise, and the similarities between the early side effects of these two agents, to our knowledge no study has investigated the role of loud noise in modulating MDMA toxicity. Therefore, in the present study, we evaluated whether cardiac effects of MDMA administration following a typical “binging” pattern are enhanced by concomitant exposure to loud noise. We selected low doses of MDMA in order to avoid gross morphological alterations, or lesions detectable under light microscopy. The myocardial alterations observed were visible only at the ultrastructural level. We found a dramatic enhancement of alterations in the mouse heart upon MDMA administration during loud noise exposure. Remarkably, this enhancement was evident both as a decrease in the threshold dose of MDMA necessary to alter the myocardial ultrastructure, and as an increase in myocardial alterations produced by a higher dose of MDMA. *Anat Rec* 267:37–46, 2002. © 2002 Wiley-Liss, Inc.

Key words: amphetamines; ecstasy; MDMA; heart; drugs of abuse

3,4-Methylenedioxymethamphetamine (MDMA, or “ecstasy”) is an amphetamine derivative that produces both acute (mainly cardiovascular) and chronic (mainly neurotoxic) effects in several animal species, from rodents to primates (including humans) (Ricaurte et al., 1988a, b, 1992; McCann et al., 1998). Long-term effects range from neurological deficits (McCann et al., 2000) to psychiatric syndromes (McGuire and Fahy, 1991; McCann and Ricaurte, 1992; McGuire et al., 1994). In the last decade, MDMA has largely been abused in conditions of elevated temperature, crowding, and high levels of noise.

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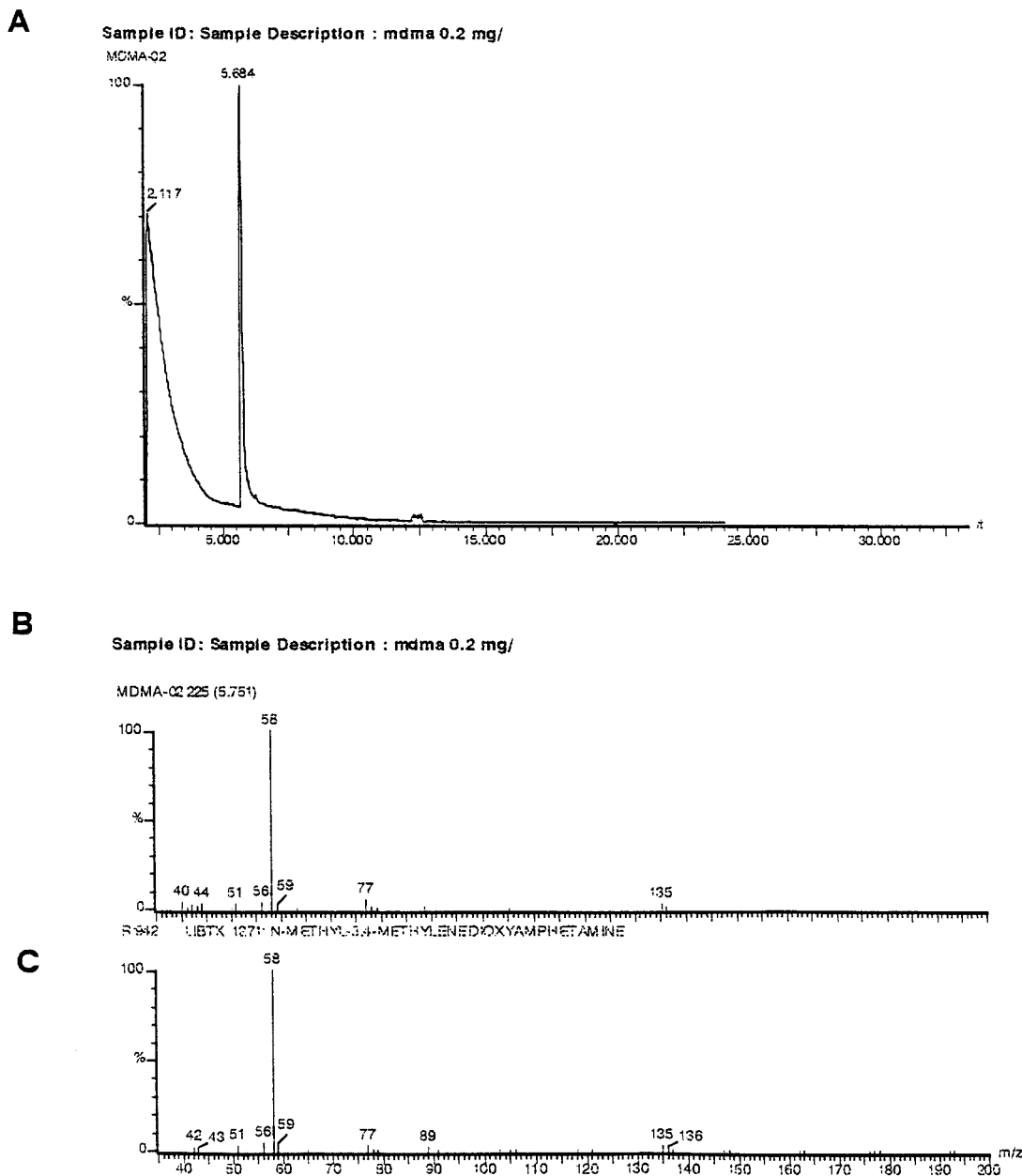


Fig. 1. GLC mass-spectrometry of MDMA crystallized from solid samples. **A:** GLC image showing a single peak with a retention time of 5.684 min, corresponding to purified MDMA. The **(B)** mass spectra of the purified compound overlaps **(C)** the MDMA spectrum obtained from the NIST library.

Although early toxicity occurring during or immediately after MDMA administration may induce dramatic and unpredictable effects (i.e., there is no clear dose-response effect (Burgess et al., 2000)), the subcellular mechanisms remain largely unknown.

This lack of knowledge is partly due to the scarcity of experimental studies investigating the early toxicity of MDMA. Studies of chronic neurotoxicity have focused on the activity of MDMA in the central nervous system (CNS), demonstrating that this neurotoxin produces a dose-dependent release of 5-hydroxytryptamine (5HT)

and dopamine (DA) (Schmidt, 1987; Battaglia et al., 1988; Ricaurte et al., 1988a, b; Steele et al., 1989; Morgan, 1999). These biochemical effects appear to be responsible for delayed neurotoxicity via the production of free radicals and reactive oxygen species (Battaglia et al., 1987; Colado et al., 1997; Fornai et al., 2001a, b).

Central monoamines may contribute to early toxicity following MDMA administration. For instance, the release of both 5HT and DA within the CNS seems to be responsible for a marked increase in body temperature, which can lead to acute hyperthermia and even death (Green et al., 1995).

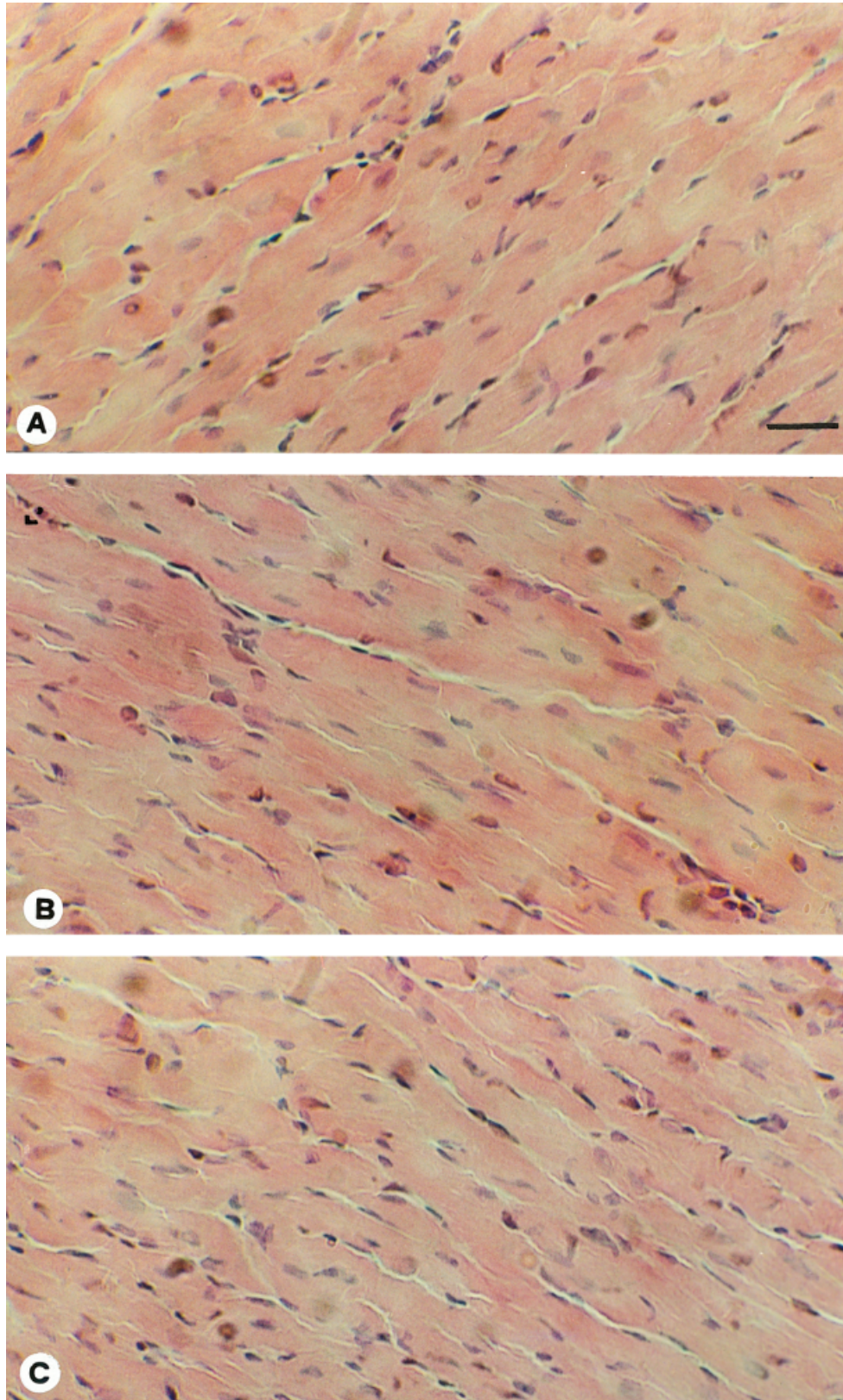


Fig. 2. Light microscopy of the myocardium. Hematoxylin-eosin-stained sections (8 μm thick) of atrial tissue from (A) a control mouse, (B) a mouse exposed to the high dose (30 mg/Kg \times 4, 2 hr apart) of MDMA, and (C) a mouse exposed to loud noise combined with the high dose of

MDMA. No morphological alterations were detected. The typical pattern of myofibrils is evident without alterations and, most remarkably, with no zones of myocytolysis. Bar = 0.05 μm .

Post-mortem studies have also demonstrated that MDMA produces rhabdomyolysis (Screaton et al., 1992), which affects the myocardium (myocytolysis) (Milroy et al., 1996) and represents a major component of early intoxication.

Even though extensive studies have been performed on the CNS, it is difficult to explain the early peripheral symptoms that are responsible for sudden death. Involvement of the peripheral serotonergic and dopaminergic systems does not appear to be sufficient to justify these dramatic cardiovascular effects.

A further point, which has often been underestimated, is the effect of MDMA on the noradrenergic pathways. It is notable that a dose of 40 mg/Kg is sufficient to induce a marked norepinephrine (NE) release, which, in turn, is followed by NE depletion. This effect occurs at the levels of both the brain and the heart (Steele et al., 1989).

In line with this, MDMA's effects on the cardiovascular system, such as tachycardia, hypertension, and arrhythmia, are present to a variable extent in humans abusing ecstasy (Downing, 1986). All of these effects might be explained by sustained NE release in both the myocardium and peripheral blood vessels. Cardiovascular side effects occurring soon after MDMA ingestion often remain unpredictable and do not appear to follow a dose-response pattern. For instance, Henry et al. (1992) concluded that occurrence of early toxicity is not attributable to an overdose of the drug. Other authors have pointed out that the same dose of MDMA that is harmless for the majority of abusers has in a few cases led to sudden death (Wolff et al., 1995). Given the lack of an established dose-response effect, the unpredictable outcome of ecstasy intake cannot be explained only by variations in the concentration of authentic MDMA sold on the illegal market (Burgess et al., 2000). A possible explanation may be derived from the fact that environmental variables play a role in modifying effects of MDMA. For instance, Albers and Sonsalla (1995) reported that MDMA toxicity depends critically on room temperature, and other studies have demonstrated an analogous influence of crowding (Wagner et al., 1981; Seiden and Ricaurte, 1987; Vargas-Rivera et al., 1990).

These environmental conditions are often present during recreational MDMA ingestion. While this finding seems to be relevant to the induction of neurotoxicity, the relationship with cardiotoxicity is far from clear. For instance, high temperature leads to a decrease rather than an increase in blood pressure, which should reduce rather than increase cardiac overload.

An in-depth analysis of the environmental pattern of ecstasy intake clearly reveals that in most cases this substance is abused in the presence of loud noise, which may affect the myocardium.

Loud noise is known to modify the heart rate (Linden et al., 1985), to increase peripheral vascular resistance (Bach et al., 1991), and to raise blood pressure (Sawada, 1993), thereby producing effects similar to those observed after ecstasy intake (see above). Apart from these functional consequences, we have demonstrated that noise exposure provokes morphological alterations in the rat myocardium (Paparelli et al., 1995; Soldani et al., 1997) which are related to increased NE innervation (Paparelli et al., 1992; Breschi et al., 1994).

Despite the frequency of concomitant exposure to ecstasy and loud noise, as well as the similarities in early side effects between these two agents, to our knowledge no

study has investigated the role of loud noise in modulating MDMA toxicity. It is crucial to analyze this point in the search for a potential synergism sustaining cardiotoxicity.

In the present study, we specifically evaluated whether loud noise enhances the cardiac effects of MDMA when administered following a typical "binging" pattern (Ricaurte et al., 1988a).

MATERIALS AND METHODS

Male C57 black mice (C57BL/6J), 8–9 weeks old, were obtained from Harlan Industries (San Pietro al Natisone, Italy). Mice were kept under controlled conditions (12-hr light/dark cycle, with lights on from 07:00 to 19:00 hr) and were fed and allowed to drink water ad libitum. Animals were handled in accordance with the Guidelines for Animal Care and Use developed by the National Institutes of Health. As the toxicity of amphetamine derivatives is highly variable, and is critically dependent on room temperature (Albers and Sonsalla, 1995) and the number of animals per cage (Fornai et al., 2001b), we carefully kept a constant room temperature (21°C) and took measures to prevent overcrowding.

Twenty-four hours before treatment, all the mice were housed one per cage (cage size: 11 cm × 10 cm × 15 cm high), allowing the mouse to move freely inside so as reduce the risk of restraint stress in all groups. Animals were assigned to the following treatment groups, each composed of 10 animals:

- Group A: control mice.
- Group B: noise-exposed mice.
- Group C: mice given MDMA (30 mg/Kg × 2, 2 hr apart).
- Group D: noise-exposed mice given MDMA (30 mg/Kg × 2, 2 hr apart).
- Group E: mice given MDMA (30 mg/Kg × 4, 2 hr apart).
- Group F: noise-exposed mice given MDMA (30 mg/Kg × 4, 2 hr apart).

When animals received combined treatment (noise + MDMA, groups D and F), the first injection of MDMA was administered at the beginning of noise exposure. The following MDMA injections were carried out at 2-hr intervals either during noise exposure (second and third injections) or at the time-point when loud noise was stopped (fourth injection).

Fig. 3. Effects of various doses of ecstasy and/or loud noise on the atrial ultrastructure. **A:** Controls show well-preserved mitochondria, atrial granules, sarcoplasmic reticulum, and myofibrils. **B:** After noise exposure, a few mitochondria show matrix dilution (arrows). **C:** Following a low dose (30 mg/Kg × 2, 2 hr apart) of MDMA, atrial ultrastructure does not differ from controls. **D:** Atrial tissue after combined exposure to loud noise and the low dose of MDMA (see part C) exhibits a typical ultrastructure, which is similar to that in B but contains nonsignificant ultrastructural alterations. **E:** Some altered mitochondria (arrows) are present in atrial sections from mice treated with the high dose (30 mg/Kg × 4, 2 hr apart) of MDMA. **F:** Several altered mitochondria (arrows) between the myofibrils are visible after loud noise combined with the higher dose (see E) of MDMA. cap, capillary blood vessel; f, myofibrils; g, atrial granules; i, intercalated discs; l, lipid droplets; m, mitochondria; sr, sarcoplasmic reticulum. Bar = 4 μm.

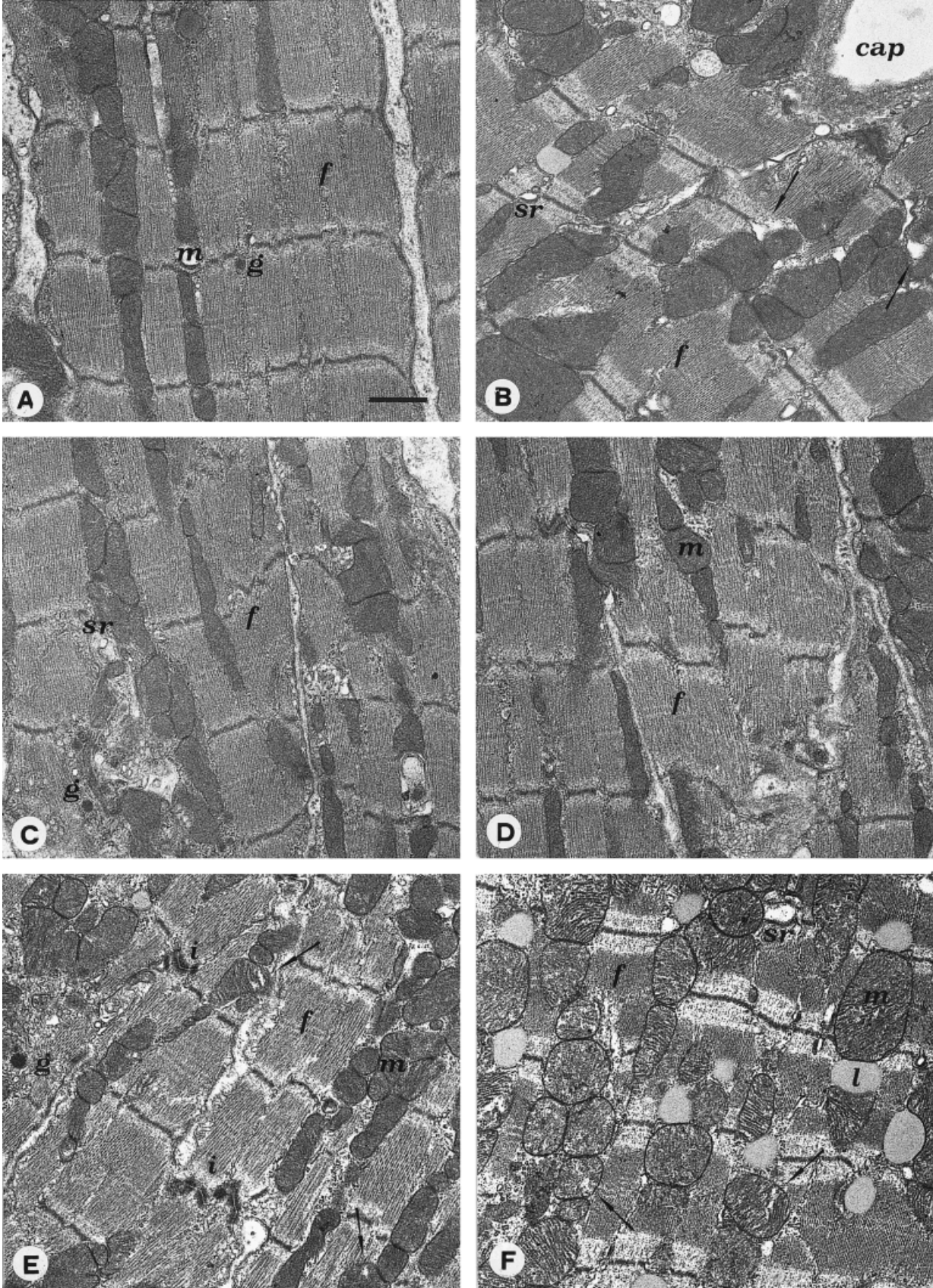


Figure 3.

TABLE 1. Effects of combined exposure to MDMA and loud noise on mitochondria in the mouse myocardium

	Controls	Noise	MDMA low	Noise + MDMA low	MDMA high	Noise + MDMA high
Atrium	2.9 ± 0.9	10.3 ± 2.3	7.2 ± 2.0	13.0 ± 2.4	5.8 ± 1.0	41.1 ± 5.8*
Ventricle	3.8 ± 0.6	14.1 ± 2.0	8.4 ± 3.5	16.5 ± 3.3*	15.8 ± 3.0	23.23 ± 3.6*

Numbers indicate the percentage mean ± SEM of altered mitochondria obtained for each treatment group (N = 10). Noise refers to mice exposed for 6 h to 100 dBA; MDMA low corresponds to a dose of 30 mg/Kg × 2, 2 hr apart; MDMA high corresponds to a dose of 30 mg/Kg × 4, 2 hr apart. The total number of mitochondria was 6,000 for each group. Mitochondria were classified as altered when matrix dilution, cristolysis and enlargement between the outer and the inner membrane were evident (see Fig. 5).

* $P < 0.01$ compared with controls.

Group A and group B received saline solution intraperitoneally (i.p.) following the same protocol used for MDMA-treated mice.

Noise continued for 6 hr and was produced by two loudspeakers (15 W), driven by a white-noise generator (0–26 kHz), which were installed 30 cm apart, on opposite sides of the cage. The noise level was set at 100 dBA and, as monitored by a sound-level meter (Quest Electronics 215), it was uniform throughout the cage.

We obtained authentic MDMA hydrochloride from solid samples ground to a powder and dissolved with diluted hydrochloric acid as previously described (Dal Cason, 1989), and modified it by repeating the crystallization procedure twice. The purity of the chloride-containing crystals was verified prior to the onset of the experiments by measuring the melting point and by running gas liquid chromatography (GLC) coupled with mass spectrometry, which demonstrated the identity and purity of the MDMA (Fig. 1).

At the end of treatment, all animals were anesthetized with ether. To obtain an optimal preservation of the heart structure, and to rule out potential artifacts, animals were perfused through the left ventricle, starting with a pulse of heparin through a 19-gauge needle. After a quick rinse with saline solution (0.9%), the heart was injected with the fixing solution (1.25% glutaraldehyde in 0.08 M cacodylate buffer and 0.03 M CaCl₂, pH 7.4). An incision was made into the lateral part of the right atrium to allow the solution to leave the blood vessels.

Light Microscopy

Since autopsies performed in people who died during MDMA intoxication often reveal cardiac myocytolysis, we first analyzed the histological preservation of the myocardium in the heart, including the same areas where electron microscopy was carried out. This was done through routine histological procedures carried out on 8- μ m-thick sections stained with hematoxylin-eosin.

Transmission Electron Microscopy (TEM)

Samples from the right atrium (at the lateral side of the vena cava opening) and the right ventricle (near the apex), less than 0.5 mm³, were further immersed in 3% glutaraldehyde fixing solution for 60 min. Specimens were post-fixed for 2 hr at 4°C in 1% buffered OsO₄, dehydrated in ethanol, and embedded in Epon-araldite. Sections were then observed under a Jeol JEM 100 SX transmission electron microscope.

From each mouse, two tissue blocks were chosen at random and 10 electron micrographs were examined at a final magnification of ×10,000. The extent of damage was

measured by counting the altered mitochondria out of the total mitochondria, and expressed as percentage values.

To avoid experimental bias, we followed the optimal procedure for electron microscopy of the myocardium (Tomanek and Karlsson, 1973; Marino et al., 1983; Soldani et al., 1997; Lenzi et al., 1998).

Statistical Analysis

Results were calculated as means ± SEM for each group. Comparisons between groups were carried out using one-way analysis of variance (ANOVA) using specialized software (Statview® for McIntosh). The null hypothesis was rejected when $P < 0.01$.

RESULTS

Light Microscopy

No morphological alterations were detected in any of the animal groups when the myocardium was observed at the microscopic level, even following the highest dose of MDMA (30 mg/Kg × 4, 2 hr apart, Fig. 2B) and combined treatment. This was confirmed by the observation of hematoxylin-eosin sections (Fig. 2A–C) showing unaltered cardiomyocytes with a typical myofibrillary pattern, without any zone of alterations as in control myocardium. Most remarkably, neither MDMA alone nor the combined treatments produced myocytolysis.

Electron Microscopy

Right atrium. The cellular ultrastructure of the control animals (group A) exhibited a typical pattern: sarcoplasmic reticulum, atrial granules, and intermyofibrillary mitochondria with a dense matrix and cristae (Fig. 3A).

Fig. 4. Effects of various doses of ecstasy and/or loud noise on the ventricular ultrastructure. **A:** The ventricular ultrastructure from controls shows well-preserved mitochondria, sarcoplasmic reticulum, and myofibrils. **B:** After noise exposure, a number of altered mitochondria showing matrix dilution are visible (arrows). **C:** When the low dose (30 mg/Kg × 2, 2 hr apart) of MDMA is administered, the ultrastructure of the cardiac ventricles does not differ from controls. **D:** In contrast, when the low dose of MDMA is administered in combination with loud noise exposure, a significant number of altered mitochondria (arrows) are observed in the ventricular ultrastructure. **E:** A higher dose of MDMA (30 mg/Kg × 4, 2 hr apart) produces several altered ventricular mitochondria in which the matrix is diluted (arrows) (see Table 1). **F:** When such a dose of MDMA is combined with loud noise, the ventricular ultrastructure is markedly affected, with a large number of altered mitochondria (arrows) appearing between the myofibrils (see Table 1). f, myofibrils; l, lipid droplets; m, mitochondria; sr, sarcoplasmic reticulum. Bar = 4 μ m.

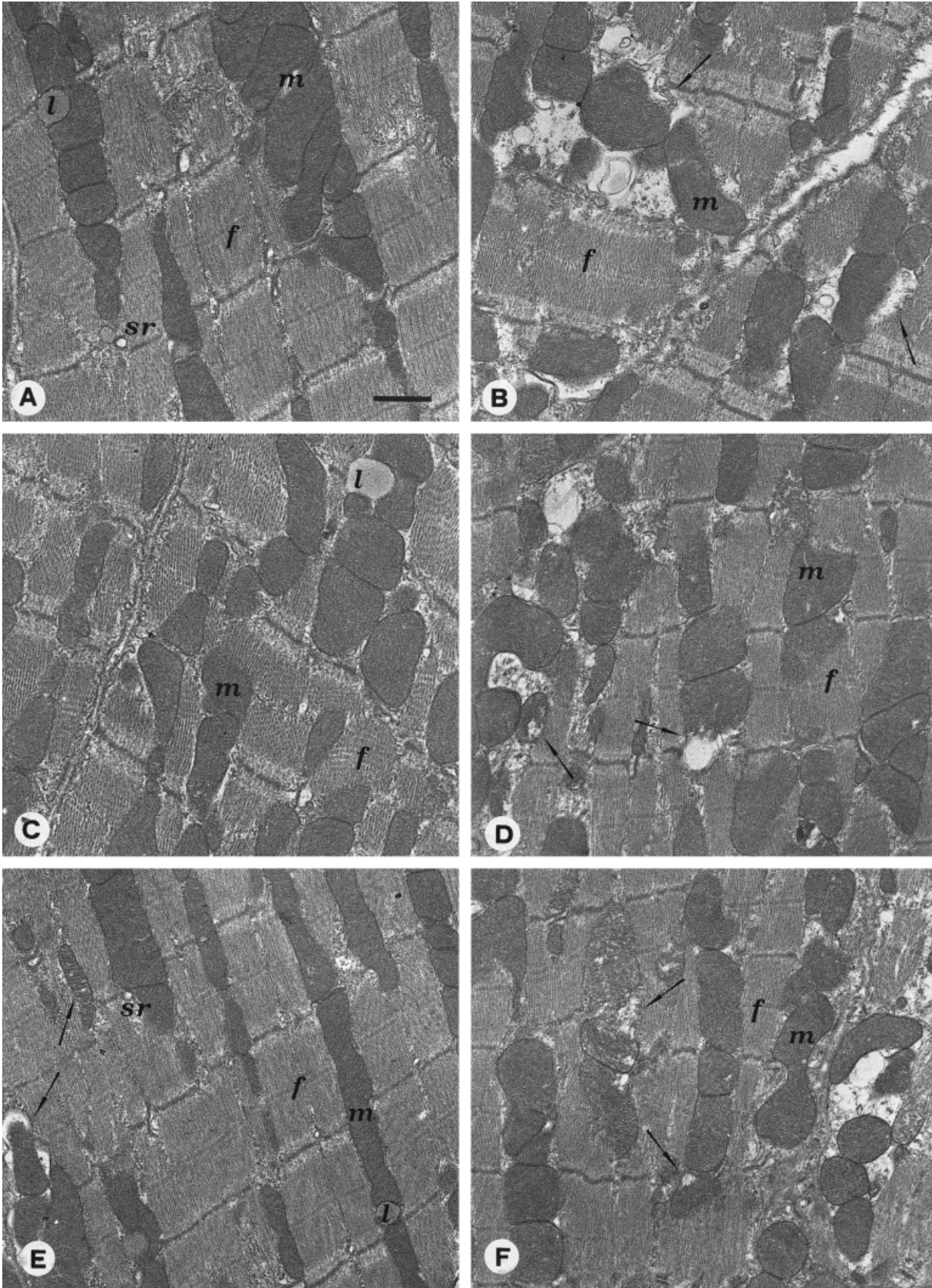


Figure 4.

Mice exposed to loud noise for 6 hr showed mild, nonsignificant ultrastructural alterations at the mitochondrial level, i.e., swelling of the membranes and dilution of the matrix (Fig. 3B; Table 1).

The atrial tissue from mice administered low doses of MDMA exhibited a normal ultrastructure (Fig. 3C), in which only a few mitochondria showed damage similar to that observed in the noise-exposed mice. The same phenomenon was observed in atrial tissue when higher doses of MDMA were administered alone (Fig. 3E).

The cardiomyocytes of mice that received a low dose of MDMA during loud noise exposure were not significantly different from controls (Fig. 3D; Table 1). When higher doses of MDMA were administered in combination with loud noise (group F), a significant alteration, and at times a dramatic effect were observed in the atrial mitochondria (Figs. 3F and 5A; Table 1). Additionally, there was a significant difference in the number of altered mitochondria when mice undergoing noise exposure combined with the highest dose of MDMA were compared with those receiving a combination of noise with a low dose of MDMA (Table 1). Remarkably, this dose dependency was not detected when mice receiving administration of different doses of MDMA alone (Groups C and E) were compared.

Right ventricle. The ventricles of control animals showed a normal ultrastructure, in which intermyofibrillary mitochondria with a dense matrix were evident (Fig. 4A), while the cardiomyocytes of noise-exposed mice (Fig. 4B) revealed nonsignificant mitochondrial alterations that were similar to those described in atrial tissue (Table 1). Similarly, the ultrastructure of ventricles from mice treated with low and high doses of MDMA revealed nonsignificant mitochondrial alterations (Fig. 4C and E; Table 1). By contrast, significant amounts of altered mitochondria were observed in ventricles from mice that received combined exposure to ecstasy to noise (at both low and high doses) and loud noise, in comparison with controls (Figs. 4D and F, and 5B; Table 1).

Although the morphology of the mitochondria was altered by combined treatments, no change in the total number of mitochondria was detected after some of the treatments. In particular, in controls the number was 21.6 ± 2.1 per μm^2 and 19.8 ± 1.8 per μm^2 in atrial and ventricular tissue, respectively, whereas in mice treated with the highest dose of MDMA alone or combined with noise exposure the number of mitochondria was 20.2 ± 1.4 per μm^2 and 20.2 ± 1.6 per μm^2 in atrial and ventricular tissue, respectively. As expected, the lower doses of MDMA alone or combined with loud noise did not modify the total number of mitochondria in the atrium or the ventricle.

DISCUSSION

The present study was motivated by previous findings showing that loud noise and MDMA, separately, produce various effects on the cardiovascular system. Given the frequency of association between MDMA and loud noise, we considered it crucial to evaluate the effects provoked by noise exposure in animals injected with MDMA.

The results of these experiments indicate a dramatic increase in ultrastructural alterations in the mouse heart upon MDMA administration during loud noise exposure. Remarkably, this was evident as a decrease in the thresh-

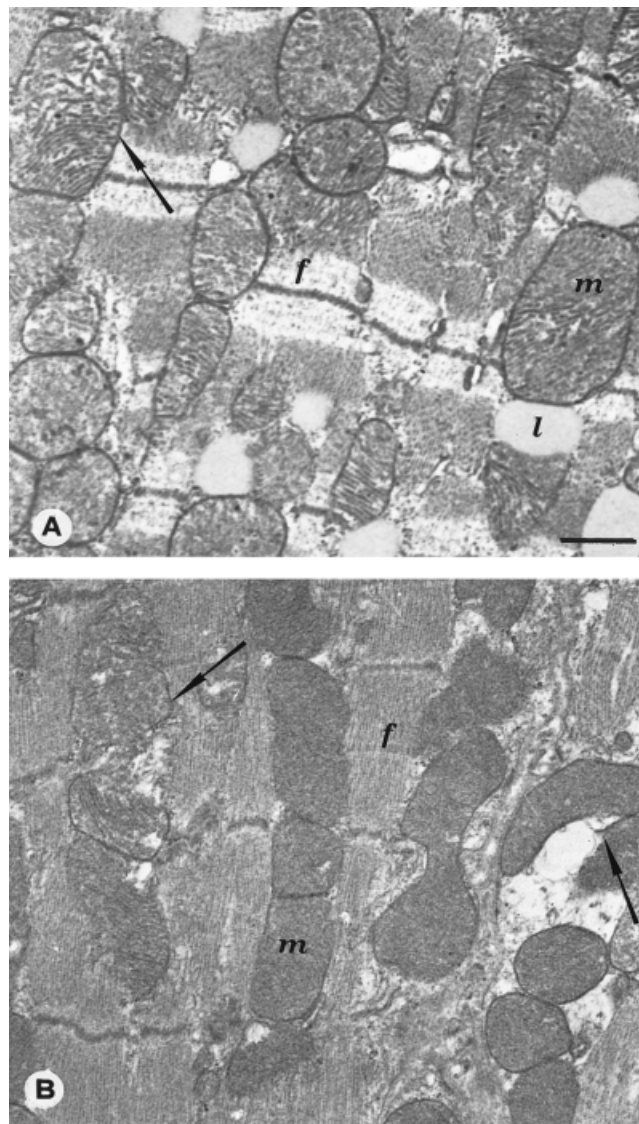


Fig. 5. Effects of combined exposure to ecstasy and loud noise on the atrial and ventricular ultrastructures at higher magnification. The (A) atrial and (B) ventricular tissues are shown in Figures 3F and 4F, respectively. The higher magnification allows detailed evaluation of mitochondrial alterations (arrows). f, myofibrils; l, lipid droplets; m, mitochondria. Bar = 2 μm .

old dose of MDMA necessary to derange the myocardial ultrastructure.

In the present study, we selected low doses of MDMA in order to avoid gross morphological alterations, or lesions that were detectable under light microscopy. The myocardial alterations observed were visible only at the ultrastructural level. In this way it was possible to detect, among various components of myocardial cells, which structure (presumably representing the primary target) was more sensitive to MDMA administration. Therefore, the study was focused on detecting subtle changes occurring at the myocardial level during MDMA exposure, and establishing whether concomitant exposure to loud noise could have modulated these effects.

As reported above, under the light microscope we observed no modification in the myocardium after MDMA or loud noise exposure. Nonetheless, both agents were seen to have modified the myocardial structure when this was examined under TEM.

This finding confirms that the experimental model selected allows evaluation of early interactions occurring between loud noise and MDMA at the subcellular level.

We found that, in line with their mechanisms of action, ecstasy and loud noise produce similar ultrastructural effects. In particular, the myocardium of mice injected with MDMA alone revealed a small number of altered mitochondria, showing a dilution of the matrix and cristolysis in both the atria and the ventricles. The same phenomenon was observed in noise-exposed mice.

On the other hand, the major finding of this study is that MDMA administration occurring during noise exposure results in the enhancement of myocardial damage compared with the consequences of MDMA or loud noise when administered separately.

Furthermore, addition of a constant level of noise exposure during ecstasy intake resulted in a dose dependency for ultrastructural alterations induced by MDMA.

The enhancement of myocardial ultrastructural damage occurred selectively through an increase in altered mitochondria.

These findings suggest that a mitochondrial dysfunction may be the first step in the toxic myocardial cell death (myocytolysis) observed in autopsies of people who died after ingesting ecstasy. Indeed, both mitochondrial alterations and myocytolysis have been reported to occur after a catecholamine-induced increase in cytosolic calcium concentration, and they can be reproduced by administering agonists for NE receptors as well (Rona, 1985). In line with this, we recently reported that loud noise determines an increased myocardial calcium entry (Salvetti et al., 2000), and that morphological changes of mitochondria are associated with mitochondrial calcium deposits (Gesì et al., 2000). The mechanisms by which this common effect (i.e., increased calcium entry) occurs remain speculative; however, it is likely that the increase in heart NE release might be at least one common final pathway for MDMA and loud noise.

In keeping with this, carrier-mediated uptake of MDMA appears to be required in order to produce NE release in the heart (Steele et al., 1989), thus confirming that NE terminals are targeted by the drug. Similarly, loud noise increases heart NE innervation (Paparelli et al., 1992), and variations in the expression of NE receptors have been described after loud noise exposure (Breschi et al., 1994). These considerations strongly suggest a common final pathway between MDMA administration and loud noise exposure in modifying the heart ultrastructure.

The relevance of the present findings to human intoxication requires further study; however, it should be considered that the estimated recreational dosage of MDMA in humans is around 3–5 mg/Kg (Green et al., 1995), which is very close to the dosages that produce toxicity in rodents (Burgess et al., 2000).

However, interspecies comparisons should not just be based on dose per body weight, since it is well known, for instance, that rodents are fast metabolizers, while primates possess an increased susceptibility to toxicity induced by MDMA (Ricaurte et al., 1988a). Additionally, previous studies in humans have not focused on the con-

comitant level of environmental noise during the intake of small amounts of the drug.

Incidentally, the present study also offers the first comparison of the effects of loud noise between different species. Although we previously reported that noise exposure results in significant mitochondrial alterations in the rat myocardium (Soldani et al., 1997), this is the first investigation of the effects of loud noise exposure on the mouse heart. This last point is very interesting in the light of similarities between mouse and human hearts (see below). Our previous studies in the rat demonstrated that loud noise induces myocardial damage, which is more pronounced in the atrium than in the ventricle; conversely, in this study (carried out in the mouse), we observed a slight increase in the number of altered mitochondria, which occurred in similar extents in the atrium and the ventricle. These latter findings led us to conclude that the mouse myocardium is more resistant to the effects induced by loud noise than its rat counterpart. This higher resistance may be due to the zonal pattern of NE receptors, which should be the final effectors for noise-induced myocardial alterations. Rats, in particular, show a marked difference in the amount of alpha-1 receptors between the atrium and ventricle (Steinfath et al., 1992). This difference is absent in mice and humans, wherein the amount of alpha receptors is barely detectable (Steinfath et al., 1992). Alpha-1 receptors produce, via hydrolysis of phosphatidylinositol, a marked increase in intracellular calcium concentration (Lazou et al., 1994). These differences may account for both the higher overall sensitivity of the rat myocardium as compared with that of the mouse, and a higher vulnerability of rat compared with mouse atria.

This point may justify variations in adrenoceptor-mediated myocardial stimulation. As stated by Endoh et al. (1991), differences in alpha-1 adrenoceptor activation among various mammalian species are responsible for interspecies variability in intracellular calcium concentrations. This might explain the differences found in myocardial consequences following sympathetic stimulation, which are particularly conspicuous after MDMA and loud noise exposure.

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