Loud Noise Enhances Nigrostriatal Dopamine Toxicity Induced by MDMA in Mice

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ABSTRACT The neurotoxicity of 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) has been intensely investigated due to the widespread abuse of this drug and its neurotoxic effects. In mice, MDMA neurotoxicity has been demonstrated for striatal dopamine (DA) terminals. However, the current literature has reported great variability in the effects induced by MDMA; this is partially due to changes in environmental conditions. For instance, elevated temperature and a crowded noisy environment markedly increase the neurotoxic effects induced by MDMA. The environmental factor loud noise is often present during ecstasy intake; however, only a few studies have analysed the consequence of a concomitant exposure to loud noise and ecstasy intake. In the present experimental work, we investigated whether nigrostriatal DA toxicity occurring after MDMA administration was potentiated in the presence of loud noise (100 dBA). We administered MDMA to C57/Black mice using a “binging” pattern for two durations of white noise exposure. We found a marked enhancement of MDMA toxicity (7.5 mg/Kg \times 4, 2 hours apart, i.p.) in the presence of white noise exposure lasting for at least 6 hours. The striatal damage was assessed by assaying DA levels as well as the loss of tyrosine hydroxylase (TH) and the increase in striatal glial fibrillary acidic protein (GFAP) immunohistochemistry. Since loud noise often accompanies ecstasy intake, the present findings call for more in-depth studies aimed at disclosing the fine mechanisms underlying this enhancement.

INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA, “ecstasy”) is an amphetamine derivative producing peripheral and central toxic effects, which were studied in several animal species, including rats (Battaglia et al., 1987; Commins et al., 1987; Schmidt, 1987; Stone et al., 1986, 1987), mice (Fornai et al., 2001, 2002, 2003; Gesi et al., 2002a, b; Schmidt and Taylor, 1988; Steele et al., 1989), guinea pigs (Battaglia et al., 1988), monkeys (Ricaurte et al., 1988a, b), and humans (McCann et al., 1998; Morgan, 1999). The relevance of these studies was confirmed by clinical investigations showing neurological deficits (McCann et al., 2000) and psychiatric syndromes (McCann and Ricaurte, 1992; McGuire et al., 1994; McGuire and Fahy, 1991) in MDMA abusers.

The mechanisms underlying MDMA toxicity remain partly unknown. In addition, the presence of a number of environmental factors, like temperature and a crowded noisy environment, are known to affect MDMA toxicity (Chance, 1946, 1947; Gunn and Gurd, 1940). This is critical since the recreational intake of MDMA occurs in the presence of elevated temperatures in a crowded environment (Henry et al., 1992). Since the environmental context plays a significant role, the deleterious effects of elevated temperature and a crowded noisy environment for humans abusing ecstasy should be taken into account. Recreational abuse of ecstasy frequently takes place in the presence of high temperature and loud music. Since temperature has already been investigated, we evaluated the effects of loud noise at a constant temperature.

Another environmental variable that often accompanies recreational ecstasy intake is loud music (Henry et al., 1992). In fact, it is a common habit among young people to ingest ecstasy while listening to hammering musical rhythms at rave parties.

To our knowledge, no previous study has examined the effects of loud noise on the neurotoxicity induced by ecstasy intake. This led us to investigate whether adding different amounts of loud noise to different doses of ecstasy (administered in a “binging” pattern) might have an additive effect on neuronal toxicity. In particular, we focused on the nigrostriatal dopamine system, which was demonstrated to be a major target of MDMA neurotoxicity in mice (Fornai et al., 2002, 2003) and we compared these dopamine (DA) system effects with potential alterations in the serotonin pathways (which,
conversely are more affected in the rat (Stone et al., 1987). Neurotoxicity was evaluated combining the non-specific expression of glial fibrillary acidic protein (GFAP) with specific markers of DA as tyrosine hydroxylase (TH), DA transporter (DAT) and DA levels, and serotonin (5HT) levels.

**MATERIALS AND METHODS**

**Animals**

Male 9–10-week-old C57/Black mice (C57BL/6) were obtained from Harlan Industries (San Pietro al Natisone, Italy). Mice were kept under controlled conditions (12-hour light/dark cycle with lights on between 07:00 and 19:00) 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, critically depending on room temperature (Albers and Sonsalla, 1995) and the number of animals per cage (Fornai et al., 2001, 2002), we carefully kept a constant room temperature (21°C) and humidity (60%) both in housing and performing the experiments.

**MDMA Purification and Authenticity Assay**

We obtained authentic MDMA hydrochloride from solid samples (Institute of Forensic Toxicology, Prof. M. Giusiani). Briefly, solid samples were ground to a powder and dissolved with diluted hydrochloric acid and crystallized twice. The purity of the chloride-containing crystals was verified before the experiments by measuring melting point and running gas chromatography (GC) and mass spectrometry, which demonstrated the authenticity and purity of MDMA as recently reported (Fornai et al., 2003; Fig. 1).

**Schedule of Treatments**

Twenty-four hours before treatment, all mice were housed one per cage (size of the cage: 11 × 10 cm wide and 15 cm high) allowing the mouse to move freely inside, in order to reduce the effects of aggregation. Mice were assigned to the following groups: (1) Controls, (2) Noise, (3) Noise and MDMA, and (4) MDMA. Controls (N = 10) were left in similar cages as experimental groups.

Mice were exposed either for 2 (N = 10) or 6 hours (N = 10) to continuous white noise produced by two loudspeakers (set at 15 W, 100 dBA), driven by a white-noise generator (0–26 kHz), which was installed 15 cm apart, on opposite sides of the cage. The noise level was monitored by a sound level meter (Quest Electronics, 215), and it was uniform throughout the cage. The level of loud noise was selected in order to mimic the same intensity to which humans are exposed during recreational ecstasy intake.

MDMA, dissolved in distilled water, was administered i.p, 7.5 mg/kg either ×2 (N = 10) or ×4 (N = 10), with 2 hours of time interval between each injection. This produced an administration schedule close to a bingeing intake.

In the combined treatments, the first dose of MDMA was administered when starting noise exposure. Four groups (each, N = 10) received combined treatments (noise + MDMA), including either 2 or 4 MDMA administration along with either 2 or 6 hours of noise.
exposure. In summary, there was a total number of 8 experimental groups (4 receiving combined treatments, 2 receiving different duration of noise exposure only, 2 receiving different doses of MDMA only) and one control group.

Seven days after treatment all animals (N = 90) were sacrificed; from each group, 4 brains were processed for morphological studies while 6 were biochemically analysed for monoamine levels, as described below.

**Light Microscopy and Immunohistochemistry**

Before sacrifice each animal was anaesthetized with choral hydrate (400 mg/kg, i.p.). In order to obtain an optimal preservation of the nervous tissue and rule out potential artefacts, mice were thoracotomized and a saline solution (0.9 % NaCl) followed by a fixing solution containing 4% paraformaldehyde dissolved in PBS (pH 7.4) was injected into the left ventricle, at room temperature (21°C) and rate of 2.5 mL/min. An incision was made in the right atrium to allow the washing and fixative solutions to flow through the circulatory system. After perfusion, brains were dissected, immersed overnight in the same fixative solution, and moved to a cryo-protective solution (30% sucrose), to be finally cut with a cryostate after freezing in isopentane.

Immunohistochemistry was carried out on striatal 20-μm-thick slices. Briefly, after fixation and permeabilization with cold methanol, endogenous peroxidase activity was removed by washing for 30 min in meth-

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Fig. 2. Effects of MDMA and loud noise on striatal DA nerve terminals. High doses of MDMA (7.5 mg/Kg ×4) produce a significant decrease in TH immunostaining in mice striatum (c) compared with control (a). This effect is potentiated by 6 hours loud noise exposure (d), which per se does not produce any reduction in TH immunostaining (b). These images are representative of semi-quantitative data obtained by densitometric analysis of TH and DAT immunostaining (e,f, respectively). a-d: ×30. Inserts at high magnification in a and d: ×300. *P < 0.05 compared with control. **P < 0.05 compared with control and MDMA ×4. (Figure continued on next page.)
anol with 0.3% hydrogen peroxide. Slices were pre-blocked with normal goat serum (Vector Laboratories, Burlingame, CA) for 2 hours and then incubated for 24 hours with primary antibodies (Ab-I) against tyrosine hydroxylase (TH), DA transporter (DAT), or glial fibrillar acidic protein (GFAP) in PBS solutions containing normal goat serum. We used mouse anti-TH Ab-I (Sigma Chemical Co., St. Louis, MO) diluted 1:2,000, rat anti-DAT Ab-I (Chemicon International, Temecula, CA) diluted 1:1,000, and rabbit anti-GFAP Ab-I (Chemicon) diluted 1:400. After careful washing, sections incubated with anti-TH or anti-DAT Ab-I were incubated with biotinylated secondary antibodies (Vector), and revealed by using the Avidin-Biotin Complex kit (Vector) followed by diaminobenzidine as chromogen (Vector). Sections incubated with anti-GFAP Ab-I were processed with fluorescein-conjugated secondary antibodies (Vector). Finally, all sections were observed under a Wetzlar Orthoplan (Leitz) light microscope. We selected TH and DAT in order to visualize the integrity of DA terminals, whereas GFAP was selected as an index of reactive gliosis that accompanies the neurotoxicity to striatal axon terminals.

Biochemical Procedures and HPLC Analysis

One week after treatment, animals were killed by decapitation, their brains were removed and dissected. Striatal samples were homogenised in 0.6 mL ice-cold perchloric acid (0.1 N) and aliquots of homogenate (50 μL) were assayed for protein content (Fornai et al., 2001). After centrifugation at 8,000 g for 10 min, 20 μL of the clear supernatant was injected into an HPLC system to measure levels of DA, 5HT, and metabolites.

Locomotor Activity

In order to evaluate the behavioral effects of repeated treatments, mice were monitored 2 hours after the last MDMA/vehicle injection and/or 2 hours after the end of noise exposure. Mice were already trained during the behavioural session carried out daily for 1 week before the experiment. Frequency of open field activity and rearing was recorded in an open field box (100 × 100 cm); open field was measured as the number of squares crossed in the time unit (2 min), and in the same time interval episodes of rearing were counted. At the end of the measurement, mice were recovered into separate cages. Measurement of locomotor activity was carried out immediately after treatment and repeated up to 7 days.

Statistical Analysis

Comparisons between groups were carried out using one-way analysis of variance (ANOVA) with Sheffe’s post-hoc analysis. Values are expressed as means ± SEM of 10 determinations. Null hypothesis (H₀) was rejected when P < 0.05.

Measurement of striatal DAT and TH immunostaining was carried out by semi-quantitative densitometry (NIH Image analysis).

RESULTS

Light Microscopy

Mice exposed to loud white noise either for 2 or 6 hours possessed a marked striatal immunostaining for TH and DAT (Fig. 2), which was comparable with what was observed in controls. Similarly, in mice receiving 2 doses of MDMA alone or in combination with noise exposure, we could not find any difference compared with previous groups (not shown). In contrast, when prolonging MDMA administration up to 4 injections, a significant decrease in striatal DAT and TH immunostaining (Fig. 2) was accompanied by the observation of intense fluorescence for striatal GFAP (Fig. 3). When this dose of MDMA was combined with noise, the brief exposure did not worsen the effects obtained with MDMA alone, while the prolonged (6 hours) noise exposure resulted in a significant enhancement of striatal MDMA toxicity, as revealed by a decrease of the semiquantitative densitometry of TH and DAT immunostained striatal slices along its rostrocaudal extent (Fig. 2) and visualized also by representative fluorescent micrographs for GFAP (Fig. 3). Neither noise exposure, nor MDMA alone, nor the combined treatments produced a decrease in the cell bodies in the substantia nigra (not shown). This is in agreement with the current belief concerning the effects of MDMA, which does not produce a decrease in the number of the cell bodies in the substantia nigra.

Striatal Monoamine Levels (Fig. 4)

While the white noise did not modify striatal monoamine levels at 7 days after exposure, it produced a
further decrease in DA levels when administered for 6 hours to mice undergoing the highest MDMA administration (Fig. 4a,b). This effect was specific for the DA component since the slight decrease in 5HT levels produced by high doses of MDMA was not potentiated by concomitant exposure to loud noise up to 6 hours (Fig. 4c,d). MDMA alone (only when administered at the dose of 7.5 mg/Kg × 2, 2 hours apart) produced a robust decrease in DA levels with a slight decrease in 5HT (Fig. 4). In contrast, administration of MDMA 7.5 mg/Kg × 2, 2 hours apart, was non-effective both alone or in combination with loud noise (Fig. 4a,b). Dopamine metabolites (DOPAC and HVA) followed the same trend (not shown).

**Behavioral Observations**

Immediately after prolonged (6 hours) noise exposure there was an increase in open field activity (114.5 ± 7.5 counts/2 minutes after noise compared with 67.5 ± 5.8 counts/2 minutes of controls), which was enhanced by concomitant MDMA administration (144.0 ± 7.7 counts/2 minutes). At 7 days following treatment, while the effect of noise or MDMA alone were not different from controls, the combined administration still produced a persistent hyperlocomotion (109.8 ± 11.5 counts/2 minutes compared with 63 ± 3.8 counts/2 minutes of controls).

**DISCUSSION**

We recently demonstrated the synergism between loud noise and ecstasy at the level of the myocardium (Gesi et al., 2002a,b). In the present study, we investigated the combined effects of MDMA and loud noise in the central nervous system focusing on the nigrostriatal pathway by measuring monoamine levels and immunohistochemistry. We found that loud noise exposure combined with ecstasy (MDMA 7.5 mg/Kg × 4, 2 hours apart) produces a selective enhancement of the nigrostriatal DA damage. This was measured by decreased TH and DAT immunostaining associated with an increased GFAP expression as well as by decreased striatal DA levels. In contrast, concomitant exposure to loud noise does not worsen the serotonergic effects produced by MDMA alone. The occurrence of a deleterious effect for the DA terminals while sparing the 5HT nerve endings is in line with previous studies showing that, in mice, neurotoxicity of ecstasy is directed primarily to the nigrostriatal DA system with a variable involvement of the serotonergic compartment (Battaglia et al., 1988; Fornai et al., 2001, 2002, 2003; Schmidt and Kehne, 1990; Schmidt and Taylor, 1988; Steele et al., 1989). Our data suggest that increased striatal GFAP immunostaining might be due to the loss of striatal DA innervation. In
a previous study, Morton et al. (2001) found that another amphetamine derivative (methamphetamine) produced a robust striatal GFAP expression when it was associated with loud music. The enhanced neurotoxicity we observed following a combined administration with MDMA occurs selectively for the DA terminals and requires that loud noise is prolonged up to 6 hours, since a shorter exposure was non-effective.

Loud noise alone produces an increased locomotor activity when measured with the open field test, which was augmented in noise-exposed mice receiving ecstasy. This latter group possessed a persistent hyperlocomotion that was detectable up to 7 days following the combined administration (i.e., mice continued to possess hyperlocomotor activity even a long time after MDMA was cleared from the brain). The mechanisms underlying these behavioural effects remain obscure; however, one might speculate that the binging dosage of MDMA used in this study might have produced a cross-sensitisation of the rodents to react with higher locomotor activity to a variety of environmental stimuli.

The significance of the present study translates into the environmental context of ecstasy intake occurring in discos or at rave parties, in which loud music is a constant feature. In fact, the durations of loud noise and the binging pattern of ecstasy intake we selected to produce a neurotoxic synergism closely matches the recreational abuse.

In this study, we found that white noise needs to be prolonged up to 6 hours in order to produce a significant effect even though it is not sufficient per se to induce neurotoxicity. These findings, joined with previous evidence we obtained in the myocardium combining MDMA and loud noise (Gesi et al., 2002a,b) indicate that noise is a crucial variable for ecstasy toxicity to peripheral organs as well as the central nervous system. The mechanisms by which loud noise is respon-
sible for the enhancement of MDMA-induced neurotoxicity is rather specific since it occurs for the DA but not for the 5HT system. These results add the effects of loud noise to those induced by temperature as critical physical agents modulating MDMA neurotoxicity (Henry et al., 1992). The persistent behavioural effects as well as the biochemical mechanisms call for more in-depth studies aimed at elucidating both at the physiological and pathological level the interaction between the central auditory pathways and the nigrostriatal DA system. The present findings should be taken into account also in predicting the relevance of loud noise for the deleterious consequence of human intoxication following ecstasy intake.

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REFERENCES


