

Ascorbic Acid Prevents 3,4-Methylenedioxymethamphetamine (MDMA)-Induced Hydroxyl Radical Formation and the Behavioral and Neurochemical Consequences of the Depletion of Brain 5-HT

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ABSTRACT MDMA-induced 5-HT neurotoxicity has been proposed to involve oxidative stress due to increased formation of hydroxyl radicals. Recently, MDMA-induced 5-HT neurotoxicity has been shown to be accompanied by a suppression of behavioral and neurochemical responses to a subsequent injection of MDMA. The intent of the present study was to examine whether suppression of the MDMA-induced formation of hydroxyl radicals by an antioxidant, ascorbic acid, attenuates both the MDMA-induced depletion of 5-HT and the functional consequences associated with this depletion. Treatment of rats with ascorbic acid suppressed the generation of hydroxyl radicals, as evidenced by the production of 2,3-dihydroxybenzoic acid from salicylic acid, in the striatum during the administration of a neurotoxic regimen of MDMA. Ascorbic acid also attenuated the MDMA-induced depletion of striatal 5-HT content. In rats treated with a neurotoxic regimen of MDMA, the ability of a subsequent injection of MDMA to increase the extracellular concentration of 5-HT in the striatum, elicit the 5-HT behavioral syndrome, and produce hyperthermia was markedly reduced compared to the responses in control rats. The concomitant administration of ascorbic acid with the neurotoxic regimen of MDMA prevented the diminished neurochemical and behavioral responses to a subsequent injection of MDMA. Finally, a neurotoxic regimen of MDMA produced significant reductions in the concentrations of vitamin E and ascorbic acid in the striatum and hippocampus. Thus, the MDMA-induced depletion of brain 5-HT and the functional consequences thereof appear to involve the induction of oxidative stress resulting from an increased generation of free radicals and diminished antioxidant capacity of the brain. **Synapse 40:55–64, 2001.**

INTRODUCTION

3,4-Methylenedioxymethamphetamine (MDMA, "Ecstasy"), an amphetamine analog, is a popular recreational drug of abuse and has been shown to be potentially toxic to the serotonergic nerve terminals of the brain of rodents, nonhuman primates, and also humans (Green et al., 1995; Scheffel et al., 1998; McCann et al., 1998; Semple et al., 1999). MDMA-induced 5-HT toxicity is based on long-term biochemical effects such as a decrease in the tissue concentration of 5-HT and its major metabolite, 5-hydroxyindoleacetic acid (Stone et al., 1986; Schmidt, 1987), decrease in the activity of tryptophan hydroxylase (Schmidt and Taylor, 1987;

Stone et al., 1987), and reduction in the [³H]-paroxetine-labeled 5-HT reuptake sites (Battaglia et al., 1987). There is also evidence of decreased immunostaining of 5-HT neurons following treatment with MDMA (Commins et al., 1987; O'Hearn et al., 1988; Scallet et al., 1988).

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The long-term depletion of brain 5-HT elicited by MDMA or fenfluramine has been shown recently to be associated with functional consequences within the serotonergic system. Neurochemical, behavioral, thermal, and neuroendocrine responses to a subsequent injection of a 5-HT-releasing agent are diminished in rats treated with a neurotoxic regimen of MDMA or fenfluramine (Series et al., 1994, 1995; Baumann et al., 1998; Shankaran and Gudelsky, 1999). In addition to the apparent alteration in presynaptic 5-HT function in rats treated with a neurotoxic regimen of MDMA, such treatment also enhances the responsiveness of postsynaptic 5-HT_{1A} receptors (Aguirre et al., 1998).

The mechanism of MDMA-induced 5-HT depletion has been proposed to involve the induction of oxidative stress (Yamamoto et al., 1998; Sprague et al., 1998). MDMA produces an acute increase in the formation of hydroxyl radicals (Colado et al., 1997a, 1999a,b; Shankaran et al., 1999a,b). In addition, treatments with antioxidants (Schmidt and Kehne, 1990; Gudelsky, 1996; Aguirre et al., 1999), as well as free radical scavengers (Colado and Green, 1995), are neuroprotective against MDMA-induced 5-HT depletion.

The intent of the present study was to determine whether the administration of the antioxidant ascorbic acid suppresses the MDMA-induced formation of hydroxyl radicals, as well as the MDMA-induced depletion of 5-HT and the functional consequences associated with this depletion. The MDMA-induced increase in the extracellular concentration of 5-HT in the striatum, the 5-HT behavioral syndrome, and the hyperthermic response were determined in rats treated with a neurotoxic regimen of MDMA and in rats given ascorbic acid concomitantly with this regimen of MDMA. Although exogenous antioxidants protect against the toxic effects of MDMA, the effect of MDMA on the endogenous antioxidants of the brain has not yet been investigated. Hence, the effect of MDMA on the tissue concentrations of the endogenous antioxidants α -tocopherol (vitamin E) and ascorbic acid were also assessed.

MATERIALS AND METHODS

Animal procedures

Adult male Sprague-Dawley rats (200–275 g; Charles River, Portage, MI) were used. The animals were housed three per cage in a temperature- and light-controlled room (lights on 0600–1800) until the day of drug treatment. All procedures were in accordance with the National Institutes of Health guidelines and approved by the institutional animal care committee.

Drug treatments

The racemic mixture of MDMA hydrochloride was provided by the National Institute on Drug Abuse.

Ascorbic acid was purchased from Sigma (St. Louis, MO). Both drugs were dissolved in 0.15 M NaCl and administered i.p. in a volume of 1 ml/kg. The doses are expressed in terms of the salt.

Rats were housed two per cage at 22–24°C and randomly assigned to one of four groups that received either vehicle, ascorbic acid (100 mg/kg, i.p. every 2 h for a total of 5 injections), MDMA (10 mg/kg, i.p. every 2 h for a total of 4 injections), or a combination of both MDMA and ascorbic acid, in which ascorbic acid was given 1 h before each MDMA injection and 1 h after the last MDMA injection. The neurochemical, behavioral, and thermal responses to a subsequent injection of MDMA were assessed in separate groups of rats 1 week after drug treatment. The doses of MDMA used to elicit these responses were chosen on the basis of our previous study (Shankaran and Gudelsky, 1999).

The effect of ascorbic acid on hydroxyl radical formation and hyperthermia produced during the administration of the neurotoxic regimen of MDMA also were determined in additional groups of rats.

In vivo microdialysis procedures 2,3-Dihydroxybenzoic acid (2,3-DHBA)

The formation of hydroxyl radicals was determined by quantifying the formation of 2,3-DHBA from salicylic acid (Floyd et al., 1984). For these experiments, salicylic acid was perfused through the microdialysis probe in a manner similar to that described by Obata and Chiueh (1992) and Hammer et al. (1993). Rats were anesthetized with chloral hydrate (400 mg/kg, i.v.), and a concentric style microdialysis probe was implanted into the striatum (1.2 mm, A; 3.1 mm, L from bregma) according to the stereotaxic atlas of Paxinos and Watson (1986). The tip of the probe was 7 mm below the surface of the brain, and the probe was secured to the skull with screws and cranioplastic cement. The rats were then allowed to recover, and the microdialysis experiment was performed the following day.

The microdialysis probes for the estimation of 2,3-DHBA were constructed entirely from polyethylene and teflon tubing, in order to eliminate metal components which promote the nonspecific formation of hydroxyl radicals. The dialysis surface of the membrane (Spectra Por, 6000 MW cutoff, 210 μ m outside diameter) for the striatum was 4.5 mm in length. The in vitro recoveries of the dialysis probes were 10–15% for 2,3-DHBA and no correction was made for recoveries.

On the day of the experiment the probe was connected to an infusion pump set to deliver Dulbecco's PBS containing 1.2 mM CaCl₂ and 10 mM glucose at a rate of 1.8 μ l/min. Following an equilibration period of 1.5 h, buffer including 5 mM salicylic acid was perfused through the probe for the duration of the experiment. At least three baseline samples were obtained at 30-

min intervals, after which drug was administered and samples were obtained thereafter at 1-h intervals.

Dopamine and 5-HT

In separate groups of rats, the extracellular concentration of 5-HT was determined in the striatum 7 days following treatment with either the neurotoxic regimen of MDMA alone or in combination with ascorbic acid. The rats were implanted with a stainless steel guide cannula under ketamine/xylazine (70/7 mg/kg, i.m.)-induced anesthesia 48–72 h prior to the insertion of the dialysis probe. On the day before the dialysis experiment a concentric style dialysis probe was inserted through the guide cannula into the striatum. The coordinates for the tip of the probe were A, 1.2 mm; L, 3.1 mm; V, -7 mm from bregma according to the stereotaxic atlas of Paxinos and Watson (1986). The microdialysis probes were constructed as described previously (Yamamoto and Pehek, 1990). The dialysis surface of the membrane (Spectra Por, 6000 MW cutoff, 210 μ m outside diameter) for the striatum was 4.5 mm in length. The probe placement was verified at the end of the experiment by visual examination of brain sections. The *in vitro* recoveries of the probes for dopamine and 5-HT were 10–15% and no correction was made for recoveries. On the day of the dialysis experiment the probe was connected to an infusion pump set to deliver Dulbecco's PBS containing 1.2 mM CaCl_2 and 10 mM glucose at a rate of 1.8 μ l/min. After a 2-h equilibration period, dialysis samples were collected every 30 min. At least three baseline samples were obtained prior to drug treatment.

Biochemical measurements

The extracellular concentration of 2,3-DHBA was quantified by high-performance liquid chromatography (HPLC) with electrochemical detection. Aliquots (20 μ l) of the dialysis samples were injected onto a C18 column (3 mm, 100 $\times$ 3 mm) connected to an electrochemical detector with a glassy carbon electrode maintained at 0.55V relative to a Ag/AgCl reference electrode. The mobile phase was similar to that described by Althaus et al. (1995) and Fleckenstein et al. (1997), and was composed of 14.15 g/l monochloroacetic acid, 7% (v/v) acetonitrile, 7% (v/v) tetrahydrofuran, pH 2.4, pumped at a flow rate of 0.4 ml/min. Peak heights following injection of 20 μ l samples were recorded with an integrator and 2,3-DHBA content was calculated based on known standards. The retention time for 2,3-DHBA was approximately 9 min.

The extracellular concentration of dopamine and the tissue concentration of 5-HT were quantified with HPLC with electrochemical detection. Samples were injected onto a C18-column (5 μ m, 100 $\times$ 2 mm) connected to an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN). The extracellular

concentration of 5-HT was determined by injecting samples onto a C18 column (MD-150, 3 $\times$ 150 mm, ESA, Chelmsford, MA) connected to an electrochemical detector (Coulchem detector; ESA). The potentials of E1 and E2 on the analytical cell (model 5014B) were -150 and 160 mV, respectively. The mobile phase for both systems consisted of 35 mM citric acid, 54 mM sodium acetate, 50 mg/l of disodium ethylenediamine tetraacetate, 70 mg/l of octanesulfonic acid sodium salt, 6% (v/v) methanol, 6% (v/v) acetonitrile, pH 4.0, pumped at a flow rate of 0.4 ml/min. Under these conditions there was no interference in the analysis of 5-HT by 3-methoxytyramine. Peak heights following injection of 20 μ l samples were recorded with an integrator and the quantities of dopamine and 5-HT were calculated on the basis of known standards.

For postmortem analysis of brain 5-HT, the rats were killed by decapitation 7 days after administration of MDMA. The tissues were kept frozen (-80°C) until analyzed for 5-HT. The tissue samples were homogenized with 0.2 N perchloric acid and centrifuged for 6 min at 10,000g and an aliquot of the resulting supernatant fluid was analyzed for 5-HT. The retention times for dopamine and 5-HT were approximately 6 and 15 min, respectively.

5-HT behavioral syndrome

Seven days following the treatment of separate groups of rats with the neurotoxic regimen of MDMA alone or in combination with ascorbic acid, 5-HT behavioral syndrome was assessed according to the methods of Spanos and Yamamoto (1989). The following components of the 5-HT syndrome were evaluated: forepaw treading, headweaving, and low body posture. On the day of the experiment the rats were placed individually in clear plastic cages and allowed to acclimate for 1 h. The rats were injected i.p. with 15 mg/kg MDMA and 5-HT behavioral syndrome was observed and rated for a 1-min period every 10 min for a duration of 90 min after MDMA administration. The dose of MDMA was selected based on previous work (Shankaran and Gudelsky, 1999). The scale used for rating was: 0 = absent; 1 = occasional (the behavior is present for <30 sec during the 1-min observation period); 2 = frequent (the behavior is present for >30 sec during the 1-min observation period); and 3 = constant (the behavior is present for the entire 1-min observation period). The scores for each behavior were summed for the nine observation periods.

Body temperature measurements

Rectal temperatures were recorded in a temperature-controlled (26°C) room in which the animals were habituated for 1 h prior to the start of the experiment. Rectal temperatures of rats were recorded during the administration of the neurotoxic regimen of MDMA

alone or in combination with ascorbic acid using a telethermometer and a thermistor probe. Measurements were taken at 1-h intervals beginning 2 h prior to the administration of MDMA. In separate groups of rats, the hyperthermic response to MDMA (7.5 mg/kg, i.p.) was determined 7 days following the treatment of rats with a neurotoxic regimen of MDMA or its vehicle in combination with ascorbic acid or its vehicle. The dose of MDMA was chosen on the basis of previous work (Shankaran and Gudelsky, 1999). Measurements were taken every 20 min for a 1-h period prior to and a 2-h period following the administration of MDMA.

Assay of endogenous antioxidants

Groups of rats were treated with either MDMA (10 mg/kg i.p., every 2 h for a total of four injections) or vehicle. The animals were killed at either 2, 6, or 12 h after the last injection of MDMA. Samples of the striatum and ventral hippocampus were dissected from 1.1 mm brain sections that corresponded to A: 1.6 to 0.5 mm and -4.9 to -6.0 mm, according to the atlas of Paxinos and Watson (1986) and stored at -80°C until analysis.

The tissue concentrations of vitamin E and ascorbic acid were quantified by HPLC with electrochemical detection based on the methods of Takeda et al. (1996) and Pachla and Kissinger (1979), respectively. For the analysis of vitamin E, the tissue samples were weighed and homogenized in ethylacetate and centrifuged at 10,000g for 6 min. An aliquot (20 μ l) of supernatant was injected onto a C18 column (250 $\times$ 4.6 mm, 5 μ m) connected to an analytical cell (model 5011) at 0.5V and a coulometric detector (ESA, Bedford, MA). The mobile phase was composed of 40 mM sodium perchlorate and 96% (v/v) methanol at a flow rate of 1.0 ml/min. For the analysis of ascorbic acid, the tissue samples were weighed and homogenized in 0.1 N perchloric acid and centrifuged at 10,000g for 6 min. Aliquots of the supernatant were injected onto a C18 column (75 $\times$ 4.6 mm, 3 μ m) connected to an electrochemical detector with a glassy carbon electrode maintained at 0.55V relative to a Ag/AgCl reference electrode. The mobile phase was composed of 24 mM sodium acetate, 0.33% glacial acetic acid, 6% (v/v) methanol, 3 mM tridecylamine, pH 4.5, at a flow rate of 1.0 ml/min.

Statistical analysis

Analyses of 2,3-DHBA and dopamine in dialysis samples were performed using a two pretreatment $\times$ two treatment $\times$ nine interval (repeated measures) ANOVA (SAS, Cary, NC). Differences between treatment groups were determined with the use of Duncan's test. The tissue concentrations of 5-HT, the scores for each component of the behavioral syndrome, and the maximal increases in body temperature elicited by a challenge injection of MDMA were evaluated using a

two pretreatment $\times$ two treatment ANOVA (Sigma-stat, Jandel Scientific, Sausalito, CA). Analysis of 5-HT in dialysis samples was performed using a four treatment $\times$ seven interval repeated measures ANOVA. Multiple pairwise were performed with the use of Student-Newman-Keuls test. Hyperthermia produced during the administration of the neurotoxic regimen of MDMA was assessed using a two treatment $\times$ eight interval repeated measures ANOVA. Multiple pairwise comparisons were performed using Student-Newman-Keuls test. The tissue concentrations of vitamin E and ascorbic acid were analyzed by Student's *t*-test. Treatment differences for all the data were considered significant at $P < 0.05$.

RESULTS

Effect of MDMA on brain ascorbic acid and vitamin E concentrations

The effects of MDMA on the tissue concentration of the endogenous antioxidants ascorbic acid and vitamin E in the striatum and hippocampus were investigated. The concentrations of vitamin E and ascorbic acid were decreased significantly ($P < 0.05$) in both brain regions 6 h after the last of the four injections of MDMA (Fig. 1). However, the concentrations of neither vitamin E nor ascorbic acid in the striatum were altered 2 or 12 h after the last injection of MDMA; the tissue concentration of ascorbic acid in the hippocampus was significantly ($P < 0.05$) reduced 12 h after the last injection of MDMA (data not shown).

Effect of ascorbic acid on MDMA-induced hydroxyl radical formation, dopamine release, and hyperthermia

The generation of hydroxyl radicals was estimated in animals during the administration of the neurotoxic regimen of MDMA in conjunction with ascorbic acid. The repeated administration of MDMA (10 mg/kg, i.p., every 2 h for a total of four injections) produced a sustained and significant (main drug effect, $F(1,20) = 43.93$, $P < 0.0005$) increase in the extracellular concentration of 2,3-DHBA in the striatum as compared to vehicle-treated animals (Fig. 2). However, the MDMA-induced increase in the extracellular concentration of 2,3-DHBA was significantly attenuated in animals treated with ascorbic acid (100 mg/kg, i.p., every 2 h for a total of five injections) concomitantly with MDMA (main drug effect, $F(1,20) = 27.49$, $P < 0.0005$).

During the administration of the neurotoxic regimen of MDMA, the extracellular concentration of dopamine in the striatum was increased significantly (main drug effect, $F(1,20) = 70.53$, $P < 0.0005$) (Fig. 3). The administration of ascorbic acid together with MDMA did not alter the MDMA-induced increase in the extracellular concentration of dopamine in the striatum.

During the treatment of rats with the neurotoxic regimen of MDMA, there was a sustained increase in

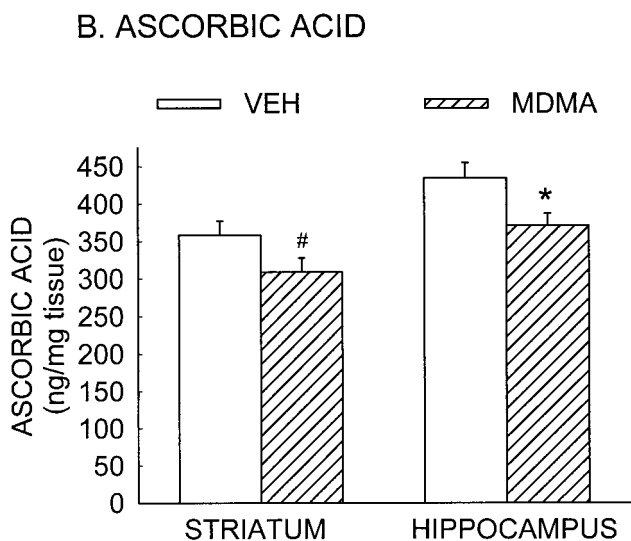
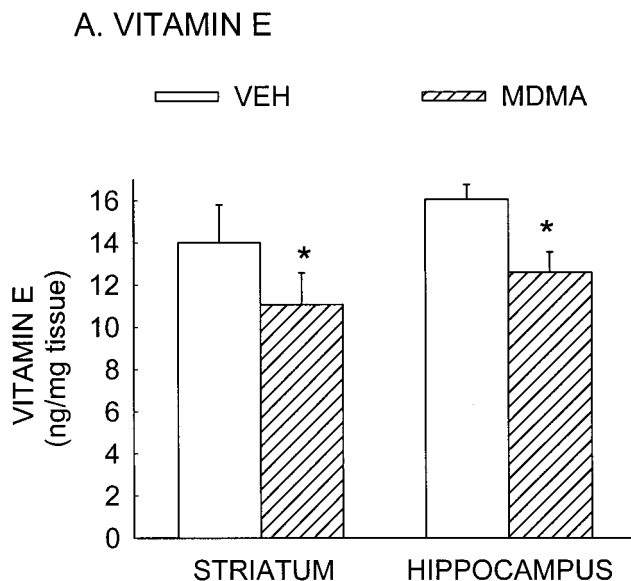


Fig. 1. Effect of MDMA on the tissue concentration of vitamin E (A) and ascorbic acid (B) in the striatum and hippocampus. Vehicle (VEH) or MDMA (10 mg/kg, i.p.) was administered every 2 h for a total of four injections. The rats were killed by decapitation 6 h after the last injection. Each value represents the mean $\pm$ SE of 12–14 rats. * $P < 0.05$ (two-tailed *t*-test) compared with vehicle-treated animals. # $P < 0.05$ (one-tailed *t*-test) compared with vehicle-treated animals.

body temperature of approximately 2.5°C and the acute hyperthermic response to MDMA was not altered by the concomitant administration of ascorbic acid (data not shown).

Effect of ascorbic acid on the MDMA-induced depletion of brain 5-HT

The administration of the neurotoxic regimen of MDMA (10 mg/kg, i.p., every 2 h for a total of four

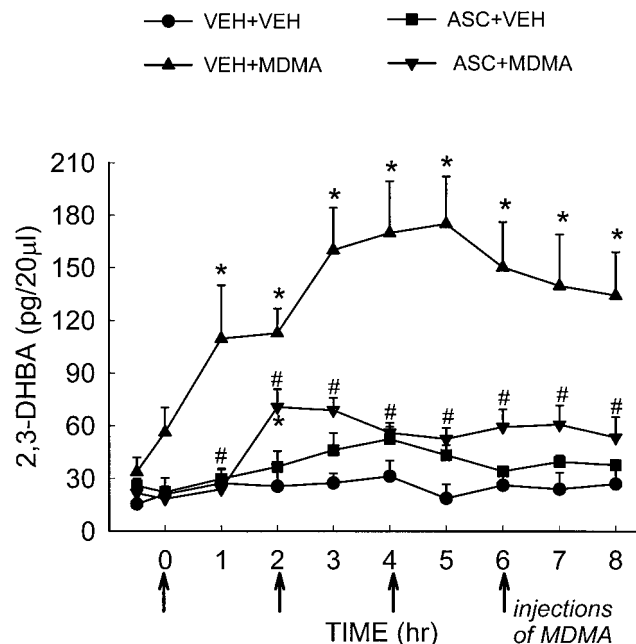


Fig. 2. Effect of MDMA on the extracellular concentration of 2,3-DHBA in the striatum of rats treated with ascorbic acid. MDMA (10 mg/kg, i.p.) was injected at time 0, 2, 4, and 6 h, as indicated by the arrows. Ascorbic acid (100 mg/kg, i.p.) was injected 1 h before each MDMA injection and 1 h after the last MDMA injection. Perfusion with dialysis buffer containing 5 mM salicylic acid commenced 1.5 h before the first injection of MDMA. The values represent the mean $\pm$ SE of 6–7 rats. *Indicates values that differ significantly ($P < 0.05$) from those of the vehicle (VEH)-treated animals. #Indicates values that differ significantly ($P < 0.05$) from those of VEH+MDMA-treated animals.

injections) produced a significant ($P < 0.05$) decrease in the concentration of 5-HT in the striatum (Table I). The tissue concentration of 5-HT in rats that received concomitant injections of ascorbic acid and MDMA was significantly greater than that in rats that had received MDMA alone. Ascorbic acid prevented the MDMA-induced depletion of striatal 5-HT to the extent that there was no significant difference in the striatal concentration of 5-HT in rats treated with ascorbic acid and MDMA compared to vehicle-treated animals.

Effect of ascorbic acid on the functional consequences of MDMA-induced 5-HT depletion

The acute administration of MDMA (7.5 mg/kg, i.p.) produced a significant increase in the extracellular concentration of 5-HT in the striatum (Fig. 4). The MDMA-induced increase in the extracellular concentration of 5-HT in the rats treated 1 week earlier with the neurotoxic regimen of MDMA was significantly less than that in rats treated with vehicle (main drug effect, $F(3,32) = 4.88$, $P < 0.01$). However, the increase in extracellular 5-HT elicited by MDMA (7.5 mg/kg, i.p.) in rats that received a combination of ascorbic acid and the neurotoxic regimen of MDMA was significantly greater than that in rats treated with the neurotoxic regimen of MDMA alone (main drug effect, $F(3,32) =$

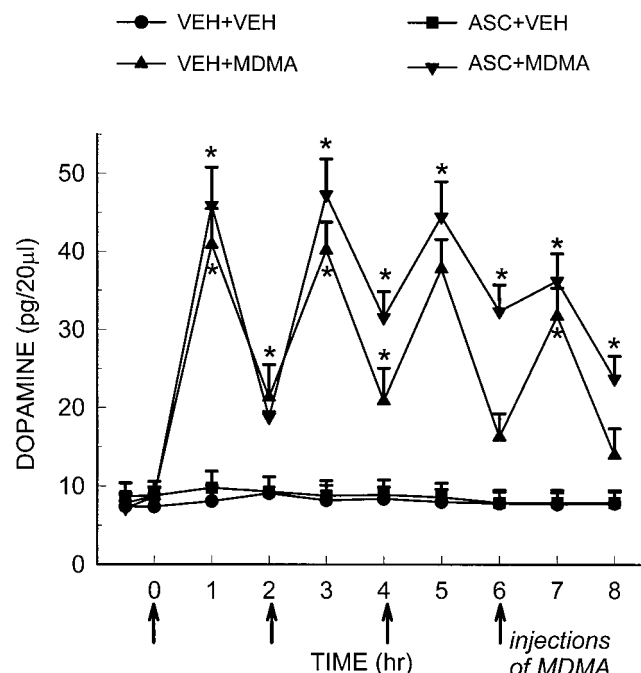


Fig. 3. Effect of MDMA on the extracellular concentration of dopamine in the striatum of rats treated with ascorbic acid. MDMA (10 mg/kg, i.p.) was injected at time 0, 2, 4, and 6 h, as indicated by the arrows. Ascorbic acid (100 mg/kg, i.p.) was injected 1 h before each MDMA injection and 1 h after the last MDMA injection. The values represent the mean $\pm$ SE of 6–7 rats. *Indicates values that differ significantly ($P < 0.05$) from those of the vehicle (VEH)-treated animals.

TABLE I. Tissue concentration of 5-HT in the striatum 1 week following drug administration

Drug treatment	5-HT (ng/mg tissue)
VEH	0.51 $\pm$ 0.05
MDMA	0.28 $\pm$ 0.03 ^{a,b}
ASCORBIC ACID	0.45 $\pm$ 0.05
ASCORBIC ACID+MDMA	0.48 $\pm$ 0.04

The rats were injected with vehicle (VEH) or MDMA (10 mg/kg, i.p.; every 2 h for a total of four injections). Ascorbic acid (100 mg/kg, i.p.) was injected 1 h before each MDMA injection and 1 h after the last MDMA injection. All animals were killed 7 days after drug administration. The values represent the mean $\pm$ SE of 6–8 rats. Multiple pairwise comparisons were performed with Student-Newman-Keuls test.

^aValue that differs significantly ($P < 0.05$) from the VEH-treated group.

^bValue that differs significantly ($P < 0.05$) from the ascorbic acid+MDMA group.

4.88, $P < 0.01$) and, moreover, did not differ significantly from the increase elicited in control animals.

The 5-HT behavioral syndrome produced by an acute injection of MDMA (15 mg/kg, i.p.) consisted of three behaviors: headweaving, forepaw treading, and low body posture (Fig. 5A–C). Each of these behaviors was suppressed significantly ($P < 0.05$) in rats treated 7 days earlier with the neurotoxic regimen of MDMA. However, the components of the 5-HT behavioral syndrome in rats that received the combined treatment of ascorbic acid and MDMA were significantly ($P < 0.05$) greater than those in rats that received the neurotoxic regimen of MDMA alone.

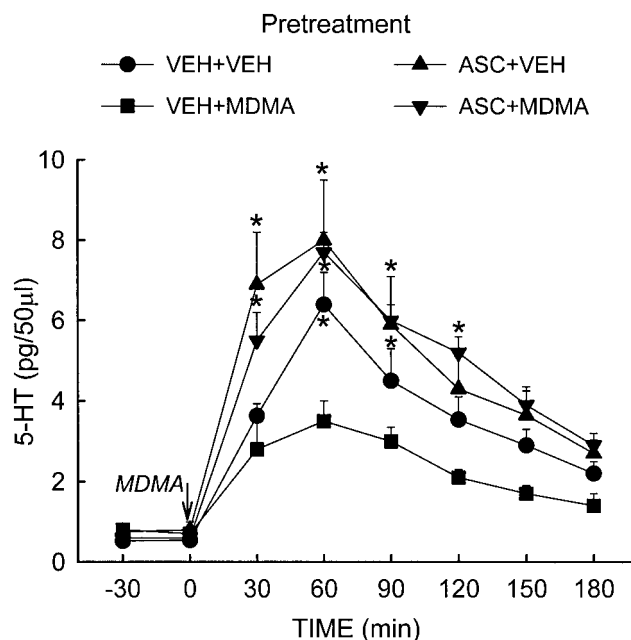


Fig. 4. Effect of ascorbic acid in combination with a neurotoxic regimen of MDMA on the subsequent MDMA-induced increase in the extracellular concentration of 5-HT in the striatum. The four groups consisted of rats that received the following treatments: vehicle only (VEH+VEH), ascorbic acid and vehicle (ASC+VEH), the neurotoxic regimen of MDMA (VEH+MDMA), or the concomitant administration of ascorbic acid and MDMA (ASC+MDMA). Details of the treatment regimen are given in Materials and Methods. Seven days after drug treatment, all animals were injected at time 0 with MDMA (7.5 mg/kg, i.p.) and dialysis samples of the striatum were collected every 30 min. The values represent the mean $\pm$ SE of 6–8 rats. *Indicates values that differ significantly (drug $\times$ time interaction $P < 0.05$) from MDMA-pretreated animals.

An acute injection of MDMA (7.5 mg/kg, i.p.) also produced a significant increase in the rectal temperature of rats (Fig. 6). The MDMA-induced hyperthermic response was significantly ($P < 0.05$) less in rats treated 7 days earlier with a neurotoxic regimen of MDMA. However, the hyperthermic response in rats that received ascorbic acid combined with the neurotoxic regimen of MDMA was significantly ($P < 0.05$) greater than that in rats that had received the treatment of the neurotoxic regimen of MDMA alone.

DISCUSSION

There is increasing evidence that oxidative stress contributes to the mechanism of the long-term depletion of brain 5-HT produced by MDMA. Gibb et al. (1990) suggested that MDMA promotes oxidative stress within 5-HT terminals on the basis of the observation that the MDMA-induced inactivation of tryptophan hydroxylase was reversed by sulfhydryl reducing conditions (Stone et al., 1989). The induction of oxidative stress by MDMA also is evidenced by the findings in the present report and earlier studies that MDMA increases the formation of hydroxyl radicals in several brain regions (Colado et al., 1997a, 1999a,b; Shanka-

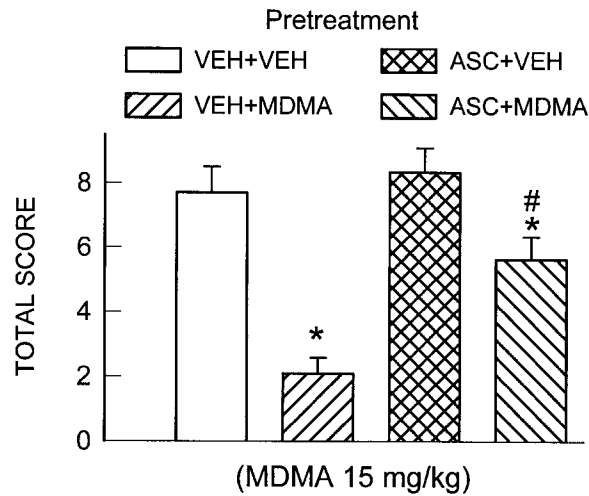
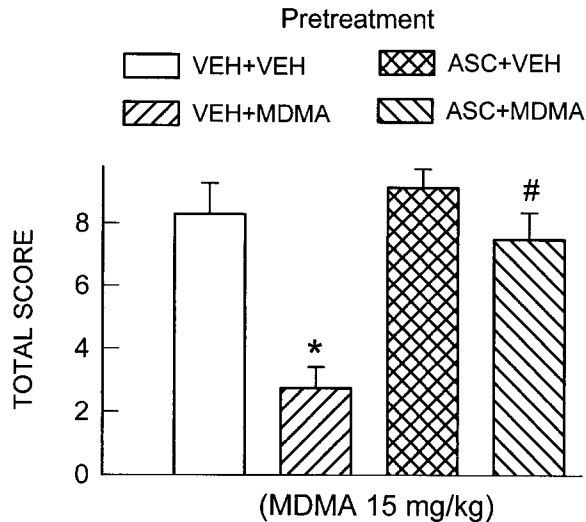
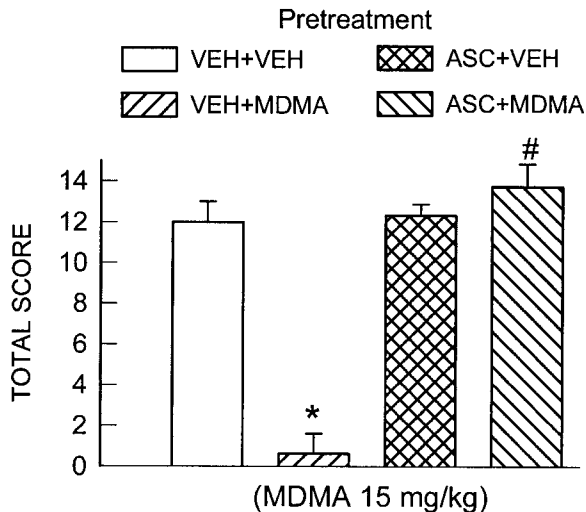
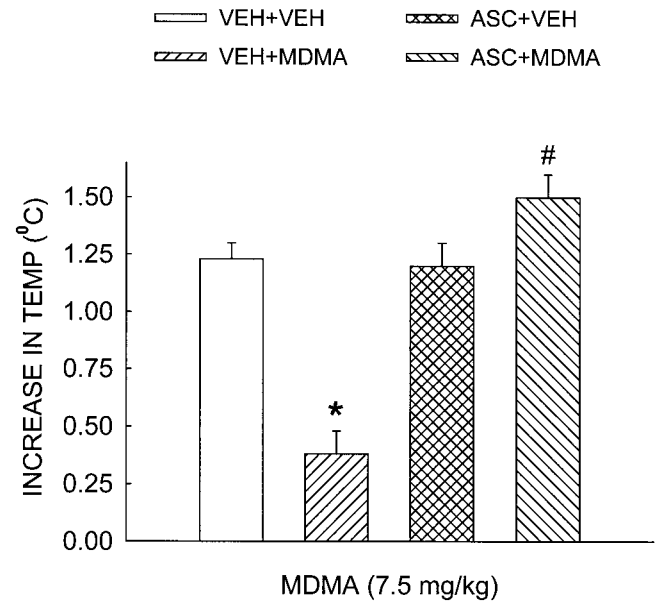
A. FOREPAW TREADING**B. HEAD WEAVING****C. LOW BODY POSTURE****Pretreatment**

Fig. 6. Effect of ascorbic acid in combination with a neurotoxic regimen of MDMA on subsequent MDMA-induced hyperthermia. Treatment groups are the same as those described in Figure 4. Seven days after drug treatment, body temperatures were recorded prior to and following the acute injection of MDMA (7.5 mg/kg, i.p.). The values (mean $\pm$ SE for 6–8 rats) represent the increase in body temperature, as calculated from the peak body temperature following MDMA treatment minus the body temperature at time 0. *Indicates a value that differs significantly ($P < 0.001$) from VEH+VEH-treated animals. #Indicates a value that differs significantly ($P < 0.05$) from the VEH+MDMA-treated animals.

ran et al., 1999a,b). In addition, MDMA has been shown to produce cellular damage, e.g., lipid peroxidation, protein nitration) consistent with the formation of free radicals (Sprague and Nichols, 1995; Colado et al., 1997b; Nixdorf et al., 2000). Finally, antioxidants (e.g., ascorbic acid, lipoic acid, cysteine) and free radical scavengers (e.g., phenyl butyl nitrone) have been shown to prevent the depletion of brain 5-HT by MDMA, as well as by other psychostimulants (Schmidt and Kehne, 1990; Gudelsky, 1996; Colado and Green,

Fig. 5. Effect of ascorbic acid in combination with a neurotoxic regimen of MDMA on the 5-HT behavioral syndrome elicited by subsequent MDMA treatment. Treatment groups are the same as those described in Figure 4. Seven days after drug treatment, the animals received an injection of MDMA (15 mg/kg, i.p.) and each component of the syndrome (i.e., forepaw treading, headweaving, and low body posture) was rated for a 1-min period at 10-min intervals for a duration of 90 min. The scale used for rating was: 0 = absent; 1 = occasional (the behavior is present for <30 sec during the 1-min observation period); 2 = frequent (the behavior is present for >30 sec during the 1-min observation period); and 3 = constant (the behavior is present for the entire 1-min observation period). The total score was obtained by a summation of the values for the nine observation periods. The values represent the mean $\pm$ SE of the total score for 6–8 rats. Post-hoc analyses of treatment means was performed with Student-Newman-Keuls test. *Indicates values that differ significantly ($P < 0.05$) from the VEH+VEH treated animals. #Indicates values that differ significantly ($P < 0.05$) from the VEH+MDMA-treated animals.

1995; Aguirre et al., 1999; Yeh, 1999; Steranka and Rhind, 1987; De Vito and Wagner, 1989).

Despite considerable evidence for a role of free radicals in the MDMA-induced depletion of brain 5-HT, the identity of the reactive oxygen species promoting oxidative stress remains unresolved. The involvement of hydroxyl radicals in this process is based on evidence that MDMA increases the formation of hydroxyl radicals and correlative evidence that drugs (e.g., mazedol, fluoxetine) that prevent MDMA-induced hydroxyl radical formation also prevent the MDMA-induced depletion of brain 5-HT (Shankaran et al., 1999a,b). However, Cadet et al. (1995) proposed, on the basis of studies in mice that overexpress CuZn superoxide dismutase, that superoxide radicals contribute to MDMA-induced neurotoxicity. An alternative source of free radicals and reactive quinones are metabolites of MDMA itself (Hirmatsu et al., 1990; Miller et al., 1996).

Oxidative stress can also be defined as a state of diminished antioxidant capacity. In this regard, it is noteworthy that MDMA not only increases hydroxyl radical formation but also diminishes the antioxidant capacity of the brain. In the present study, MDMA administration reduced the concentrations of ascorbic acid and vitamin E in the striatum and hippocampus. The reduced form of ascorbic acid acts as an antioxidant in the cytoplasm by scavenging hydroxyl, peroxy, and superoxide radicals (Bendich et al., 1986), and in the membrane through regeneration of oxidized vitamin E (Niki, 1987a). Vitamin E is the only lipophilic antioxidant capable of scavenging oxygen radicals in the membrane and may act to prevent lipid peroxidation (Niki, 1987b). There is evidence that the brain concentrations of these antioxidants are also altered in response to other oxidative insults such as temporary focal ischemia, treatment with MPTP, exposure to 100% oxygen, and traumatic brain injury (Karibe et al., 1994; Desole et al., 1995; Urano et al., 1997; Awasthi et al., 1997).

The long-term depletion of brain 5-HT and reduction of 5-HT uptake sites produced by MDMA has often been viewed as evidence of 5-HT neurotoxicity. However, this remains a controversial issue. An alternative view is that MDMA may produce a downregulation, rather than neurotoxicity, of 5-HT terminals. This contention is based, in large part, on the infrequency with which MDMA produces effects (e.g., silver staining, GFAP staining) that are largely viewed as indices of neurotoxicity (Slikker et al., 1988; O'Callaghan et al., 1995; Aguirre et al., 1999). It remains to be determined whether other quantifiable indices of neurotoxicity (e.g., cleaved tau) may be elicited by MDMA.

Regardless of whether MDMA produces a long-term downregulation or neurotoxicity of 5-HT terminals, such biochemical alterations of 5-HT terminals produced by MDMA are accompanied by functional conse-

quences in 5-HT neurotransmission. Recently, Shankaran and Gudelsky (1999) reported that several of the acute effects of MDMA, such as an increase in the extracellular concentration of 5-HT, the 5-HT behavioral syndrome, and hyperthermia, are blunted in rats treated previously with a neurotoxic regimen of MDMA. The results of the present study are in accord with this earlier report. Poland et al. (1997) and Series et al. (1994, 1995) have also demonstrated that the depletion of brain 5-HT by MDMA results in suppressed behavioral, neurochemical, and hormonal responses to another 5-HT releasing agent, fenfluramine. A similar blunting of hormonal responses to fenfluramine has been reported in human abusers of MDMA (Gerra et al., 1998). Furthermore, the administration of other neurotoxic psychostimulants, e.g., p-chloroamphetamine, fenfluramine, also produce diminished responses to a subsequent injection of fenfluramine (Series et al., 1994, 1995; Baumann et al., 1998).

In the present study, treatment with ascorbic acid prevented the MDMA-induced depletion of 5-HT, as well as the functional impairments associated with this depletion. Thus, it seems reasonable to conclude that the diminished functional responses that accompany a neurotoxic regimen of MDMA are a consequence of the MDMA-induced depletion of brain 5-HT.

It is not possible to completely exclude the possibility that pharmacokinetic tolerance contributes to the diminished functional responses to an acute injection of MDMA following the repeated administration of the drug. However, our previous finding (Shankaran and Gudelsky, 1999) that the acute elevation of extracellular dopamine elicited by MDMA is unaltered by the prior administration of a neurotoxic regimen of MDMA diminishes the likelihood of this possibility.

In view of the fact that ascorbic acid acts as an antioxidant capable of scavenging hydroxyl, peroxy, and superoxide radicals (Padh, 1991; Awasthi et al., 1997), the present results also provide evidence that the reversal of functional deficits by ascorbic acid is due not only to the attenuation of MDMA-induced 5-HT depletion, but also to the suppression of MDMA-induced oxidative stress.

Inasmuch as the increase in the extracellular concentration of dopamine and hyperthermia produced by MDMA have been proposed to be critical for MDMA-induced 5-HT neurotoxicity (Stone et al., 1988; Brodtkin et al., 1993; Malberg et al., 1996; Sprague et al., 1998; Colado et al., 1998; Shankaran et al., 1999a), it is noteworthy that ascorbic acid did not alter these responses during the administration of a neurotoxic regimen of MDMA. Thus, the neuroprotective effects of ascorbic acid most likely are due to its actions as an antioxidant.

It also is unlikely that the neuroprotection afforded by ascorbic acid against the MDMA-induced depletion of brain 5-HT and impairment of functional responses

was due to alterations in the pharmacokinetic disposition of MDMA in the brain. Gudelsky (1996) has shown that the administration of ascorbic acid does not alter the accumulation of MDMA in the brain, as determined by *in vivo* microdialysis.

In summary, the administration of MDMA resulted in a decrease in the concentrations of the endogenous antioxidants ascorbic acid and vitamin E and an increase in the generation of hydroxyl radicals in the brain. Ascorbic acid treatment prevented the MDMA-induced generation of hydroxyl radicals and the depletion of 5-HT, as well as the functional consequences that accompany this depletion. These data are supportive of the conclusion that the MDMA-induced depletion of 5-HT, and the functional consequences thereof, appear to involve the induction of oxidative stress resulting from an increased generation of free radicals and a decreased antioxidant capacity of the brain.

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