

Superoxide Radicals Mediate the Biochemical Effects of Methylenedioxymethamphetamine (MDMA): Evidence From Using CuZn-Superoxide Dismutase Transgenic Mice

JEAN LUD CADET, BRUCE LADENHEIM, HIROSHI HIRATA, RICHARD B. ROTHMAN, SYED ALI, ELAINE CARLSON, CHARLES EPSTEIN, AND TIMOTHY H. MORAN

Molecular Neuropsychiatry (J.L.C., B.L., H.H.) and Clinical Psychopharmacology (R.B.R.) Sections, NIH/NIDA, Intramural Research Programs, Baltimore, Maryland 21224; Neurochemistry Laboratory, National Center for Toxicological Research, Jefferson, Arkansas 72079 (S.A.); Department of Pediatrics, University of California, San Francisco, California 94143 (E.C., C.E.); and Department of Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 (T.H.M.)

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ABSTRACT The subacute and long-term biochemical effects of methylenedioxymethamphetamine (MDMA) were assessed in homozygous and heterozygous transgenic (Tg) mice that carry the complete sequence of the human copper-zinc (CuZn) superoxide dismutase (SOD) gene. Non-transgenic (Non-Tg) mice showed significant decreased in striatal dopamine (DA) and dihydroxyphenylacetic acid (DOPAC) levels both at 24 h and at 2 weeks after a single injection of MDMA (50 mg/kg). Heterozygous SOD-Tg mice showed DA depletion only at the 24 h time point. In contrast, homozygous SOD-Tg mice show no DA or DOPAC depletion at either the 24 h or at the 2 week time points. Moreover, three injections of MDMA (50 mg/kg) given 24 h apart also caused marked reduction of striatal DA and DOPAC in Non-Tg mice when these substances were measured 2 weeks after the last MDMA injection. That injection schedule also caused small decreases in DA levels in the heterozygous animals but no changes in the homozygous mice; DOPAC levels were not affected in the heterozygous nor in the homozygous SOD-Tg mice. Furthermore, the multiple injection schedule caused significant decreases in DA and DOPAC in female Non-Tg mice but not in the two strains of transgenic mice. Neither the single dose nor the multiple dose schedule of MDMA injections affected striatal serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels in any of the three strains of mice. These results support previous observations that MDMA-induced biochemical effects are observed in the DA systems of mice, whereas these effects are seen in the 5-HT systems of rats. The present observations also document for the first time a role for the production of superoxide radicals in these effects of MDMA. These mice are an important tool for dissecting pathways involved in drug-induced neurotoxicity.

INTRODUCTION

Oxidative stress and oxygen free radicals are thought to play an important role in both the acute and chronic effects of a number of neurotoxic processes. These include administration of some drugs and radiation-induced injury, as well as oxygen-induced injury to the central nervous system (Ames et al., 1993; Cadet, 1988; see Janssen et al., 1993 for a comprehensive review). Superoxide radicals and hydrogen peroxide are pro-

duced during electron transport in the mitochondria (Boveris and Chance, 1973). In addition, endoplasmic reticulum and peroxisomes, as well as nuclear and plasma membranes generate oxygen-based radicals (Freeman and Crapo, 1982). Cells protect themselves against reactive oxygen intermediates (ROI) by using

antioxidants such as the superoxide-scavenging enzyme superoxide dismutase (SOD) (Fridovich, 1986; Touati, 1988), H_2O_2 -metabolizing enzymes such as catalase and glutathione peroxidase, and other nonenzymatic agents such as glutathione, other thiols, and vitamin E (Cadet, 1988). The CuZnSOD enzyme is of interest for a number of reasons. For example, persons with Trisomy 21 have increased CuZnSOD activity in their tissues (Kedziora and Bartosz, 1988) while it has been shown that mutations in the gene that codes for that enzyme is associated with some cases of familial amyotrophic lateral sclerosis (Rosen et al., 1993).

Despite the belief that oxygen free radicals are of importance in some neuropathological states, it has not always been possible to examine their roles directly. Recently, however, transgenic animal technology has made it possible to constitutively increase the level of cytosolic CuZnSOD in mice by inserting the complete human CuZnSOD gene (Epstein et al., 1987, 1994). We have used these mice to evaluate the role of oxygen-based radicals in the toxic manifestations of methamphetamine (Cadet et al., 1994) and MPTP (Przedborski et al., 1992) and have shown that the toxic effects of these drugs were prevented in SOD-Tg mice. When taken together these results suggest that oxidative mechanisms might play an important role in the neurotoxicity of drugs that affect monoaminergic systems.

MDMA (Ecstasy) is a drug of abuse that affects the monoaminergic systems of rodents and nonhuman primates (Battaglia et al., 1987; Brodtkin et al., 1993; Commins et al., 1987; Insel et al., 1989; Johnson et al., 1988; Logan et al., 1988; O'Hearn et al., 1988; Schmidt, 1987; Stone et al., 1986). In the rat, MDMA causes marked and persistent serotonin (5-HT) depletion which is associated with 5-HT terminal cell loss (Commins et al., 1987; Schmidt, 1987). In contrast to the observations in the rat, MDMA injections given to mice cause striatal dopamine depletion without significantly affecting the serotonin system (Logan et al., 1988; O'Callaghan and Miller, 1994; Stone et al., 1987). The mechanisms involved in these neurotoxic events are not understood but might involve the production of oxygen-based radicals during the metabolic breakdown of MDMA to its catecholamine (Hiratmatsu et al., 1990). Both O_2 and H_2O_2 are known to be produced during the metabolism of catecholamine (Cohen and Heikkila, 1974; Graham, 1978). The toxic effects of these drugs might also be due to increased oxidative stress associated with the metabolic breakdown of dopamine released after MDMA administration to rodents. This reasoning is partially supported by observations that DA depletion or decreased DA synthesis cause significant reduction in the neurotoxic effects of the drug (Schmidt et al., 1991; Stone et al., 1988).

In addition to their neurotoxic effects in the brain, drugs of abuse can cause acute lethality with increasing doses of amphetamine analogs (Hardman et al., 1973).

The mechanisms for this acute toxicity are also not understood but probably involve deleterious effects on the central nervous system (Nichols et al., 1975). These could include seizures with secondary anoxic events and associated oxidative damage to the brain. In order to test that idea, we have used CuZnSOD-Tg mice to investigate their acute reactions to various doses of MDMA (Cadet et al., 1994). SOD-Tg mice were indeed protected against death caused by the drug. These results thus support the notion that superoxide radicals are involved in the acute lethal effects of the drug. In the present study, we have extended our observations to the neurotoxic effects of MDMA on the dopaminergic systems of Non-Tg and SOD-Tg mice.

MATERIALS AND METHODS

Animals

Male and female transgenic (Tg) mice of strain 218/3 carrying the complete human CuZnSOD gene were used in these experiments. These animals were produced as previously described (Epstein et al., 1987). Heterozygous and homozygous SOD-Tg mice as well as non-transgenic (Non-Tg) mice were used in these experiments. The heterozygous Tg mice had a mean 2.6-fold increase while the homozygous Tg had a mean 5.7-fold increase in enzyme activity when compared to Non-Tg mice. The animals were housed three per cage and had free access to food and water. The rooms were maintained with a 12 h light/dark cycle. All animal use procedures were according to the NIH guide for the Care and Use of Laboratory Animals and were approved by the local NIDA Animal Care and Use Committee.

Receptor autoradiographic studies

In order to evaluate if there were any differences in DA uptake sites between the three strains of mice, receptor autoradiographic studies were carried out using the cocaine analog [^{125}I]3-(4-iodophenyl)tropane-2-carboxylic acid isopropyl ester ([^{125}I]RTI-121) to label DA uptake sites that are thought to be important to the neurotoxic effects of amphetamine analogs in rodents (Marek et al., 1990). Brains from the three strains were rapidly removed and frozen in isopentane on dry ice and stored frozen at -70°C . Sections (20 μm thick) were cut at -18°C and thaw-mounted on gelatin-coated glass slides. The slides were kept at -70°C until used in the autoradiographic studies. [^{125}I]RTI-121 (SA: 2200 Ci/mmol) was used in the binding studies. Binding assays were performed according to the following protocol. Briefly, slide-mounted sections were incubated for 60 min at room temperature with [^{125}I]RTI-121 (150,000 cpm/ml) in a binding buffer (BB) consisting of 137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4 , and 10 mM NaI. Specific binding was determined in the presence of 10 μM GBR-12909 and represented greater than 90% of total binding. At the end of the incubation period, the slides were washed in fresh ice-cold buffer, dipped

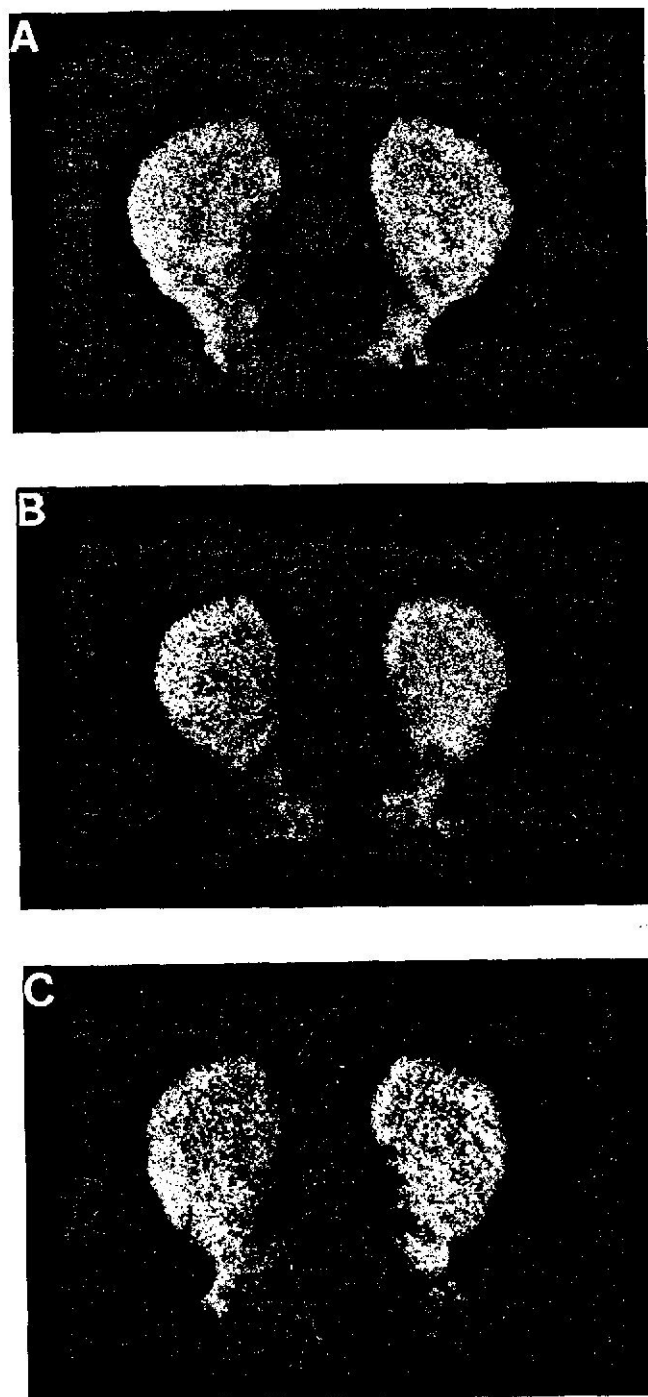


Fig. 1. Quantitative autoradiographic representation of [125 I]RTI-121 labeled striatal DA uptake sites in Non-Tg (A), heterozygous (B), and homozygous (C) male SOD-Tg mice. There were no significant differences between the three strains of mice. See Table I for quantitative values.

in ice-cold distilled water, and dried under a stream of cool air. The slides were then apposed to hyperfilms (Amersham, Arlington Heights, IL) with plastic standards ([125 I]microscales, Amersham) for 48 h at room

temperature. The films were then developed according to routine procedures. [125 I]RTI-121 binding was then quantified using a MacIntosh computer-based image analysis system (Image, NIH) using standard curves generated from the [125 I]microscales. Non-specific binding was at the level of the film background.

Drug treatment

On the day of experiments, male mice were given saline, single, or multiple doses of 50 mg/kg of MDMA via the intraperitoneal route. The animals that received a single dose of MDMA were sacrificed 24 h or 2 weeks after the drug injection. The mice that received multiple doses (50 mg/kg $\times$ 3 separated by 24 h) were sacrificed 2 weeks later. Female mice underwent the multiple dose schedule and were sacrificed 2 weeks later. On the day of sacrifice, mouse brains were rapidly removed, and different brain regions were dissected out for the measurements of DA, DOPAC, 5-HT, and 5-HIAA using HPLC techniques as described below.

HPLC

Concentrations of dopamine (DA), serotonin (5-HT), and their respective metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and 5-hydroxyindoleacetic acid (5-HIAA), were quantitated by a modified method of high performance liquid chromatography using a Bondapak C-18 column (Waters) combined with electrochemical detection (Bioanalytic Systems (BAS), W. Lafayette, IN) according to the method of Zaczek and Coyle (1982). Briefly, each brain region was weighed and diluted with 1 ml of 1% perchloric acid containing 10 ng/ml of the internal standard, 3,4-dihydroxybenzamine (DHBA). Brain tissue was then disrupted by ultrasonication and centrifuged. Aliquots (15 μ l) were injected onto the HPLC system using a Waters WISP 712 automated injection system for separation of the neurotransmitters DA, 5-HT and their metabolites, DOPAC and 5-HIAA.

Statistical analyses were done using the original biochemical data expressed as pg/mg wet weight. Comparisons were done using analysis of variance (ANOVA) followed by Fisher's PLSD using Statview 4.02 on a MacIntosh (Quadra 840 AV) computer. The null hypothesis was rejected at the 0.05 level.

RESULTS

Quantitative autoradiographic distribution of [125 I]RTI-121 in SOD-Tg mice

Figure 1 shows the autoradiographic distribution of [125 I]RTI-121-labeled DA uptake sites in the striatum of Non-Tg (A), heterozygous (B), and homozygous (C) SOD-Tg mice. Quantitative analysis showed no significant differences between the three groups of mice for the Non-Tg, SOD-Tg (hetero) and SOD-Tg (homo), respectively (Table I).

TABLE I. Striatal [125 I]RTI-121-labeled DA uptake sites in SOD-Tg mice¹

	Non-Tg	SOD-Tg (hetero)	SOD-Tg (homo)
Dorsolateral	4.54 $\pm$ 0.12	4.58 $\pm$ 0.12	4.77 $\pm$ 0.10
Dorsomedial	4.20 $\pm$ 0.08	4.01 $\pm$ 0.12	4.17 $\pm$ 0.06
Ventrolateral	4.76 $\pm$ 0.07	4.99 $\pm$ 0.11	4.97 $\pm$ 0.15
Ventromedial	4.60 $\pm$ 0.08	4.38 $\pm$ 0.10	4.39 $\pm$ 0.09

¹The values represent means $\pm$ SEM (nCi/mg tissue) of 3–5 mice per group. There were no differences between the groups. Abbreviations are hetero, heterozygous and homo, homozygous SOD-transgenic mice.

Effects of a single injection of MDMA on monoamine levels in the striatum

DA and DOPAC

A single injection of MDMA (50 mg/kg) to male mice markedly reduced striatal DA levels in the Non-Tg and in the heterozygous SOD-Tg mice. Homozygous mice were not affected (Fig. 2A). There were also significant decreases in DA levels in animals sacrificed 2 weeks after the drug administration. The decreases observed in the Non-Tg mice were comparable to those observed at the 24 h time point. However, DA values had reverted back to normal in the heterozygous mice. Again, there were no significant changes in the homozygous Tg mice.

DOPAC levels were also significantly affected by a single dose of MDMA (Fig. 2B). The pattern of changes in the Non-Tg mice was very similar to that observed for striatal DA levels, although the magnitude of decreases was smaller for DOPAC than for DA. DOPAC levels were not affected in any of the SOD-Tg strains.

5-HT and 5-HIAA

A single injection of MDMA to male mice caused no significant changes in the levels of 5-HT at either the 24 h or the 2 week time point in any of the three strains of mice (Fig. 3A). 5-HIAA levels were also not affected by the single injection of MDMA (Fig. 3B).

Effects of multiple doses of MDMA on striatal monoamine levels

DA and DOPAC

Three injections of a large dose (50 mg/kg) of MDMA to male mice cause marked reduction in striatal DA levels. Post-hoc analyses revealed that the changes corresponded to a marked decrease in DA concentration in the striata of MDMA-treated Non-Tg mice (Fig. 4A). DA levels were also affected by MDMA in the heterozygous SOD-Tg mice although the decreases observed in these mice were of smaller magnitude than those observed in the Non-Tg mice. The homozygous SOD-Tg mice were not affected. DOPAC levels were also decreased in the Non-Tg mice but not in either the heterozygous or homozygous SOD-Tg mice (Fig. 4B).

In female Non-Tg mice, the effects of MDMA on striatal DA levels were comparable to those observed in male mice (compare Fig. 4A to Fig. 5A). However, whereas male heterozygous SOD-Tg mice show significant de-

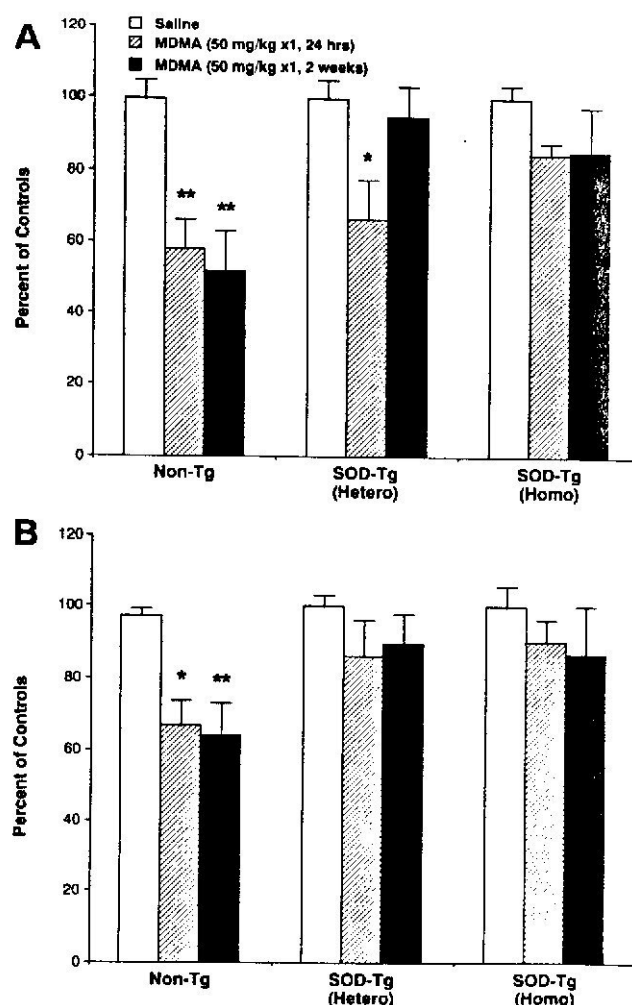


Fig. 2. CuZnSOD male transgenic mice are protected against the deleterious effects of a single dose (50 mg/kg) of MDMA on striatal DA (A) and DOPAC (B). The mice were sacrificed at the indicated times after the injection of MDMA. Values represent means $\pm$ SEM (percentage of respective controls) (n = 4–6 mice per group). There were no significant differences in DA or DOPAC levels between the saline injected groups: DA = 14.30 $\pm$ 0.54, 12.06 $\pm$.20, 12.41 $\pm$.77; DOPAC = 1.59 $\pm$ 0.06, 1.26 $\pm$.06, 1.27 $\pm$.15 (means $\pm$ SEM, ng/mg tissue) for the Non-Tg, heterozygous and homozygous SOD-Tg mice, respectively. Hetero, heterozygous; Homo, homozygous. Key: * = P < 0.005; ** = P < 0.001 vs. respective controls.

creases in striatal DA levels, such was not the case for female heterozygous mice. Striatal DA levels were not affected in homozygous female SOD-Tg mice. The decreases in DOPAC levels reflect the changes observed in DA levels (Fig. 5B).

5-HT and 5-HIAA

The three injections of MDMA caused no significant changes in the levels of 5-HT at either the 24 h or the 2 week time point in any of the three strains of male mice (Fig. 6A). 5-HIAA levels were also not affected by these injections (Fig. 6B). 5-HT and 5-HIAA were also not affected in the female mice (not shown).

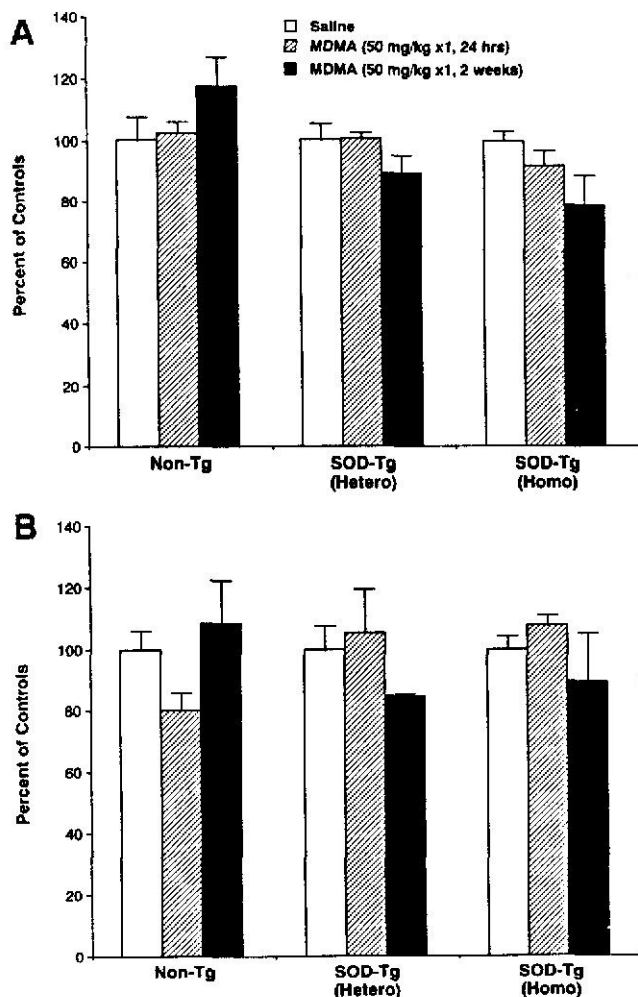


Fig. 3. A single injection of MDMA (50 mg/Kg) did not affect striatal 5-HT (A) and 5-HIAA (B) in Non-Tg, heterozygous (Hetero), and homozygous (Homo) male SOD-Tg mice sacrificed at the indicated times after the injection of the drug. Values represent means $\pm$ SEM (percentage of respective controls) ($n = 4-6$ mice per group). There were no significant differences in 5-HT or 5-HIAA levels between the saline injected groups: 5-HT = 0.62 ± 0.09 , 0.71 ± 0.02 , 0.64 ± 0.03 ; 5-HIAA = 0.42 ± 0.05 , 0.38 ± 0.02 , 0.44 ± 0.02 (means $\pm$ SEM, ng/mg tissue) for the Non-Tg, heterozygous, and homozygous SOD-Tg mice, respectively.

DISCUSSION

Our results show, as previously reported (Logan et al., 1988; O'Callaghan and Miller, 1994), that MDMA affects the DA but not the 5-HT system of mice. This is in contrast to what has been observed in other mammals (Battaglia et al., 1987; Johnson et al., 1988; Logan et al., 1988; O'Hearn et al., 1988; Stone et al., 1986). These results suggest that there are significant species differences in both the subacute and chronic effects of amphetamine analogs. This is consistent with our recent observations that methamphetamine affects the DA system of mice more than the 5-HT system (Cadet et al., 1995).

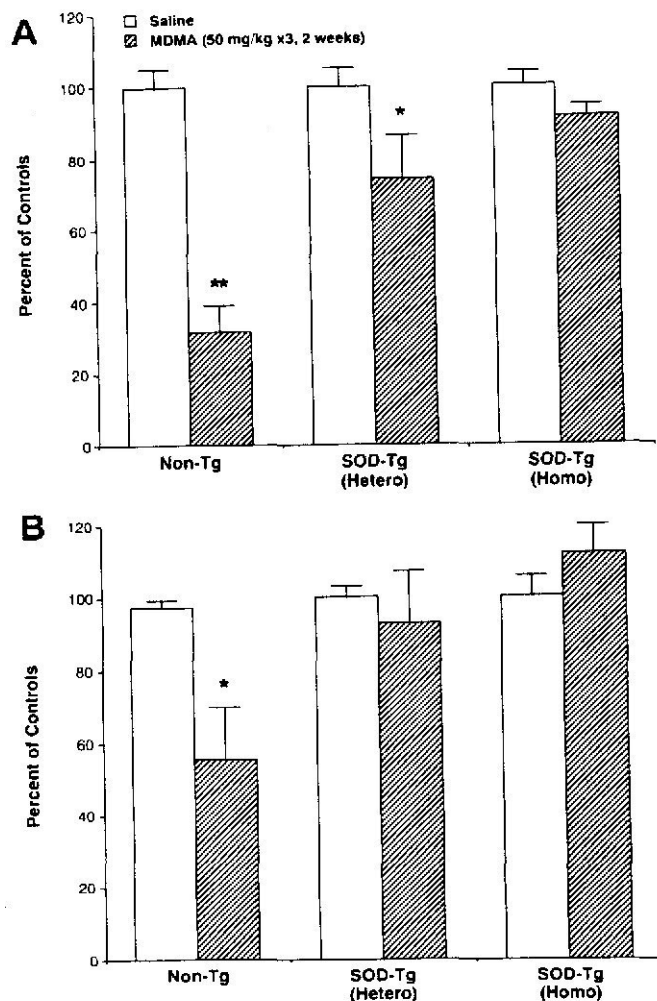


Fig. 4. CuZnSod male transgenic mice are protected against the deleterious effects of three injections of MDMA (50 mg/kg given 24 h apart) on striatal DA (A) and DOPAC (B). The mice were sacrificed two weeks after the last injection. Values represent means $\pm$ SEM (percentage of respective controls) ($n = 4-6$ mice per group). There were no significant differences in DA or DOPAC levels between the saline injected groups: DA = 13.09 ± 0.78 , 13.07 ± 0.77 , 13.10 ± 0.59 ; DOPAC = 1.59 ± 0.03 , 1.34 ± 0.05 , 1.34 ± 0.08 (means $\pm$ SEM, ng/mg tissue) for Non-Tg, heterozygous and homozygous SOD-Tg, respectively. Key: * = $P < 0.05$; ** = $P < 0.0001$ vs. respective controls.

This is the first demonstration that SOD-Tg mice that have high levels of CuZnSOD in the brain are protected against the effects of MDMA on the striatal dopaminergic system. These results are in accord with our recent demonstration that the toxic effects of methamphetamine (METH) on the DA system are attenuated in CuZnSOD-Tg mice (Cadet et al., 1994). These observations suggest that the neurotoxicity of the amphetamines and of their substituted analogs may indeed be mediated, in part, by the overproduction of the superoxide anion. This could result from the formation of 6-hydroxydopamine (6-OHDA) as suggested by Seiden and Vosmer (1984). Because the neurotoxic effects of 6-OHDA is associated with the production of the superox-

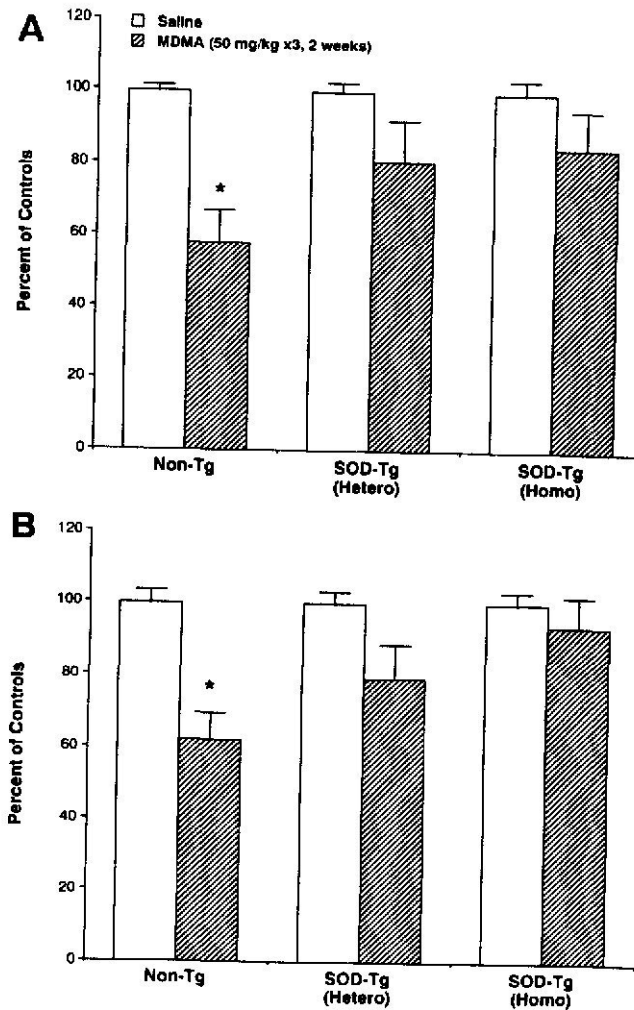


Fig. 5. CuZn SOD female transgenic mice are protected against the deleterious effects of MDMA on striatal DA (A) and DOPAC (B). The mice were sacrificed 2 weeks after the last injection. Values represent means $\pm$ SEM (percentage of respective controls) ($n = 4-6$ mice per group). There were no significant differences in DA or DOPAC levels between the saline injected groups: DA = 13.04 ± 0.20 , 14.30 ± 0.37 , 13.88 ± 0.48 ; DOPAC = 1.58 ± 0.06 , 1.47 ± 0.05 , 1.56 ± 0.05 (means $\pm$ SEM, ng/mg tissue) for Non-Tg, heterozygous, and homozygous SOD-Tg, respectively. Key: * = $P < 0.005$ vs. respective controls.

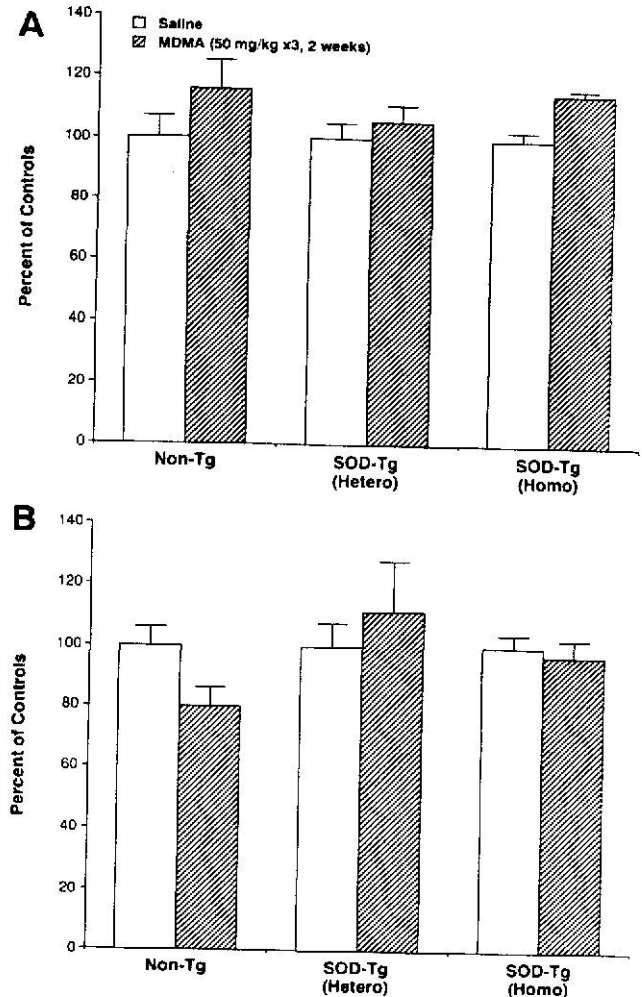


Fig. 6. Effects of three injections of MDMA (50 mg/kg given 24 h apart) on striatal 5-HT (A) and 5-HIAA (B) in Non-Tg heterozygous (Hetero), and homozygous (Homo) male SOD-Tg mice sacrificed at the indicated times after the injection of the drug. Values represent means $\pm$ SEM (percentage of respective controls) ($n = 4-6$ mice per group). There were no significant differences in 5-HT or 5-HIAA levels between the saline injected groups: 5-HT = 0.56 ± 0.05 , 0.71 ± 0.07 , 0.78 ± 0.05 ; 5-HIAA = 0.49 ± 0.03 , 0.56 ± 0.07 , 0.52 ± 0.04 (means $\pm$ SEM, ng/mg tissue); for Non-Tg, heterozygous, and homozygous SOD-Tg, respectively.

ide radical (Cohen and Heikkila, 1974), the intracellular accumulation of 6-OHDA would result in the overwhelming of the scavenging systems within striatal dopaminergic terminals. Thus, the protective effects observed in SOD-Tg mice may be due to rapid breakdown of the superoxide radicals secondary to the high levels of enzyme in these mice (Epstein et al., 1987). This proposal is supported by the recent report that methamphetamine does cause intracellular accumulation of reactive oxygen species in vitro (Cubells et al., 1994) and that MDMA increases striatal DA release after a single administration of the drug (Nash et al., 1991).

In addition to the increased production of superoxide radicals via the metabolic breakdown of released cate-

cholamines, metabolic pathways involving the formation of catecholamines from MDMA must also be taken into consideration. For example, it has been reported that MDMA is converted to dihydroxymethamphetamine (Hiratmatsu et al., 1990). This reaction is cytochrome P-450-dependent and results in a metabolite (probably a quinone) that is capable of forming an adduct with thiol compounds (Hiratmatsu et al., 1990). This oxidation reaction was inhibited by SOD (Hiratmatsu et al., 1990). If left unchecked, these reactions could lead to toxic damage in monoaminergic neurons in the fashion similar to that suggested for other catecholamines (Graham, 1978). Thus, these toxic reactions, if they occur in vivo, may be inhibited in the presence

of high levels of endogenous SOD found in the SOD-Tg mice, as observed in the *in vitro* situation in the presence of exogenous SOD (Hiratmatsu et al., 1990).

The observation that homozygous SOD-Tg mice show complete protection whereas there was some DA depletion in the heterozygous SOD-Tg mice when DA levels were measured 24 h after drug administration suggests that there might be a dose-response phenomenon so that very high levels of SOD are needed to counteract the effects of the oxidative stress that might be observed after MDMA injections. These results also suggest that only stressful events might help to uncover differences between the Non-Tg, heterozygous as well as homozygous SOD-Tg mice since there were no differences in the number of striatal [¹²⁵I]RTI-121-labeled DA uptake sites between mice that had not gotten any drug injections (see Table I). This is important since the DA system is known to play an important role in the biochemical effects of the amphetamines (Marek et al., 1990; Schmidt et al., 1991; Stone et al., 1988).

Finally, it is also of interest that female Non-Tg mice show less of a decrement in striatal DA levels than male Non-Tg mice after the administration of three injections of MDMA (compare Fig. 4 to Fig. 5). Moreover, male heterozygous mice were somewhat more affected than female heterozygous mice after receiving multiple doses of MDMA (compare Fig. 4 to Fig. 5). Thus both Non-Tg and SOD-Tg female mice show a similar pattern in their response to MDMA. These results are consistent with the gender effects on lethality that we had observed previously (Cadet et al., 1994) and provide further support for the notion of an endocrine-oxidant state interaction as previously suggested (Cadet et al., 1994). This may be related to the differences in prevalence of Parkinson's disease between men and women (Mayeux et al., 1992). Further studies will need to evaluate the relationship between gender and oxidative stress in mammalian systems. In any case, the present study does support the view that free radicals are involved in neurotoxin-induced effects on the mammalian dopaminergic systems (Cadet, 1988; Cadet et al., 1994; Cubells et al., 1994; Olanow, 1993).

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