Role of Endogenous Dopamine in the Central Serotonergic Deficits Induced by 3,4-Methylenedioxymethamphetamine[1]

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ABSTRACT

Similar to other amphetamine analogs 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy"), a currently popular illicit drug, has been characterized recently as a serotonergic neurotoxin due to its ability to cause long-lasting deficits in markers of central serotonergic function in animals. Because the serotonergic toxicity associated with the MDMA analog methamphetamine has been linked previously to endogenous dopamine and because MDMA, like methamphetamine, elicits pronounced dopamine release *in vitro*, we have examined the role of endogenous dopamine in both the immediate (3 hr) and longer-term (3 days) central serotonergic deficits induced by systemic MDMA administration to rats. Depletion of central dopamine content with α-methyl-*p*-tyrosine or reserpine, or selective destruction of nigrostriatal dopamine projections with bilateral 6-hydroxydopamine-induced substantia nigral lesions, partially blocked the immediate MDMA-induced reduction in rat striatal tryptophan hydroxylase (TPH) activity. In addition, the longer-term TPH deficits caused by a high single dose of MDMA were completely prevented by prior α-methyl-*p*-tyrosine or reserpine, and attenuated significantly by inhibition of dopamine uptake with the selective dopamine-uptake blocker GBR 12909. These results implicate endogenous drug-released dopamine as a partial mediator of the initial decrease in TPH activity caused by MDMA and as an important prerequisite to the development of long-term MDMA-induced neurotoxicity. Potential mechanisms of dopamine-mediated toxicity are discussed.

MDMA ("ecstasy") has gained recent notoriety, both as a popular recreational drug (Peroutka, 1987) and as an apparent serotonergic neurotoxin in rats. Its unique central properties in humans derive from a structural similarity to both the classical stimulant, amphetamine, and to mescaline and other hallucinogens. When administered systemically to rats, MDMA causes a rapid and persistent loss of all indices of central 5-HT function; these deficits include reductions in regional brain 5-HT and 5-HIAA content (Schmidt *et al.*, 1986; Stone *et al.*, 1986) accompanied by a decrease in the activity of central TPH (the rate-limiting enzyme for 5-HT synthesis) (Stone *et al.*, 1986; Schmidt and Taylor, 1987), histological evidence of serotonergic nerve terminal destruction within 1 day of MDMA administration (Commins *et al.*, 1987), and a long-term loss of central 5-HT-uptake sites (Schmidt *et al.*, 1987; Commins *et al.*, 1987). Taken together, these data provide evidence of a selective neurotoxic effect of MDMA on rat serotonergic pathways, similar in scope to the toxicity induced by the classical serotonergic neurotoxin, PCA (Fuller, 1978; Sanders-Bush and Steranka, 1978) and the N-desmethyl analog to MDMA, MDA (Ricaurte *et al.*, 1985; Stone *et al.*, 1986), itself a popular illicit drug. In rat brain, the serotonergic deficits resulting from multiple doses of MDMA are still apparent 110 days after treatment (Stone *et al.*, 1987); long-term effects occur in guinea pigs and primates as well (Commins *et al.*, 1987; Ricaurte *et al.*, 1987; Wilson *et al.*, 1987). The persistence of these effects in laboratory animals has raised concern over the possible toxicological consequences of illicit human use of MDMA.

Although the mechanism of the long-term serotonergic effects of MDMA and its congeners currently remains unknown, several lines of evidence argue against the possibility that the parent compound itself is the toxic agent. The ability of 5-HT-uptake inhibitors to block the development of MDMA-induced toxicity (Schmidt, 1987b) suggests that the toxic agent depends on an active uptake process to gain entrance into target neurons; however, Schmidt and co-workers (1987) were unable to demonstrate an active accumulation of MDMA within striatal synaptosomes, suggesting that the parent lipophilic drug molecule probably crosses neuronal membranes by passive mechanisms. In addition, MDMA itself has no direct inhibitory

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ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; 5-HT, 5-hydroxytryptamine (serotonin); 5-HIAA, 5-hydroxyindoleacetic acid; TPH, tryptophan hydroxylase; PCA, *p*-chloroamphetamine; MDA, 3,4-methylenedioxyamphetamine; METH, methamphetamine; DA, dopamine; AMT, α-methyl-*p*-tyrosine; TH, tyrosine hydroxylase; 6-OHDA, 6-hydroxydopamine; MDE, N-ethyl-3,4-methylenedioxyamphetamine.

effect on TPH enzyme activity *in vitro* (Schmidt and Taylor, 1987). These observations suggest the involvement of an intermediate process in MDMA-induced toxicity, possibly the formation of a neurotoxic metabolite or an imbalance in endogenous neurochemistry caused by the presence of drug in the brain.

MDMA differs from its structural relatives amphetamine and METH in that, in addition to effects on serotonergic neurons (METH being more potent than amphetamine in this regard; Peat *et al.*, 1985), these drugs cause long-term toxic damage to DA systems as well (Ellison *et al.*, 1978; Hotchkiss and Gibb, 1980; Ricaurte *et al.*, 1980). Early investigations into the mechanism of action of amphetamine linked the behavioral stimulant effects of this compound to endogenous brain catecholamines, subsequent to the discovery that amphetamine-induced behavior could be prevented by prior administration of AMT (Weissman *et al.*, 1966; Dingell *et al.*, 1967; Scheel-Krüger, 1971), an inhibitor of TH (the rate-limiting enzyme for DA biosynthesis). Additionally, catecholamine synthesis inhibition was found to block the long-term DA depletion caused by amphetamine (Fuller and Hemrich-Luecke, 1982). More recently, endogenous catecholamines have been implicated as mediators for the toxic effects of METH, not only on the dopaminergic system (Gibb and Kogan, 1979; Schmidt *et al.*, 1985) but on the serotonergic system as well (Schmidt *et al.*, 1985). Thus, inhibition of catecholamine synthesis with AMT prevents both the persistent decrease in dopaminergic variables and the long-term loss of central TPH activity and 5-hydroxyindole content induced by METH (Schmidt *et al.*, 1985). Restoration of central DA synthesis by addition of *l*-Dopa and a peripheral decarboxylase inhibitor to the above-mentioned dosing regimen, restores the ability of METH to induce toxic damage. Further support for the involvement of DA in the serotonergic effects of METH was supplied recently by Johnson and co-workers (1987b) who were able to prevent, selectively, the METH-induced decrease of neostriatal TPH activity, by prior destruction of the neostriatal DA-containing afferents with bilateral 6-OHDA injections into the substantia nigra. METH-induced decreases of TPH activity in the hippocampus and frontal cortex, two brain regions which receive dopaminergic input from areas other than the substantia nigra, were not prevented by prior nigral lesioning. Thus, protection against the detrimental serotonergic effects of METH was afforded only in those brain regions depleted previously of their endogenous DA content.

Although of lower potency, MDMA is similar to METH in its ability to elicit DA release *in vitro* (Schmidt *et al.*, 1987); additionally, systemic administration of MDMA results in a transient elevation of rat striatal DA content (Stone *et al.*, 1986; Schmidt *et al.*, 1986) suggestive of drug-induced DA release *in vivo*. Hence, analogous to the involvement of DA in METH-induced serotonergic damage, we speculated that the high local concentrations of DA resulting from presumed *in vivo* MDMA-induced DA release might play a similar role in the serotonergic deficits caused by MDMA. To test this hypothesis, we examined the effects of prior central DA depletion on both the immediate and long-term (neurotoxic) serotonergic changes induced by systemic MDMA administration.

Methods

Male Sprague-Dawley rats (200–250 g) were housed three to five per cage in a temperature-controlled (26°C) animal room with a 12-hr alternating light-dark cycle. Food and water were available *ad libitum*.

Short-term experiments. Before acute MDMA administration, groups of rats were subjected to one of four pretreatments with the aim to deplete central DA content; pretreatment consisted of: 1) systemic injection of AMT; 2) systemic injection of reserpine; 3) combined injection of AMT + reserpine; or 4) local bilateral injections of 6-OHDA into the substantia nigra. AMT-methyl ester (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.9% saline and administered i.p. (120 mg/kg, calculated as the free amino acid) 90 min before MDMA. Reserpine (Sigma Chemical Co.) was dissolved in a vehicle of 1% citric acid containing polyethylene glycol-400 (4:1, v/v) and injected i.p. (5 mg/kg) 12 hr before MDMA. Rats receiving the combined AMT-reserpine pretreatment were administered AMT (60 mg/kg) 10.5 hr after the reserpine injection (5 mg/kg) and 90 min before MDMA. Control rats for the above pretreatments received vehicle injections alone. Bilateral 6-OHDA-induced nigral lesions were performed as described by Hanson *et al.* (1981). In brief, rats were anesthetized with chloral hydrate (~375 mg/kg i.p.) and immobilized in a stereotaxic instrument. After a rostro-caudal incision in the skin overlying the skull, bilateral burr holes were drilled in the skull and 4 μl of 0.1% ascorbate saline containing 8 μg of 6-OHDA (Sigma Chemical Co.) were injected bilaterally into the substantia nigra (5.1 mm posterior to bregma; 2 mm lateral to midline; 6.6 mm ventral to the brain surface) at a rate of 1 μl/min. The needle was allowed to remain in place for 5 min after the injection before being withdrawn. Sham-lesioned animals received bilateral injections of ascorbate vehicle alone. After the injection, skull holes were filled with bone wax, the incision was closed with wound clips and rats were allowed 7 to 10 days to recover before MDMA administration.

The location of the site of injection in the substantia nigra pars compacta was verified in a representative number of animals with bilateral injections of 1 μl of methylene blue. Additionally, neostriatal TH activity was measured bilaterally with a tritiated water-release assay (Nagatsu *et al.*, 1964). Lesions were considered successful in those nigral tissues ipsilateral to neostriata exhibiting a > 80% 6-OHDA-induced reduction in TH activity (as compared to sham-lesioned controls); only these striatal tissues were examined for statistical analyses.

Acute MDMA treatment consisted of a single s.c. injection of *dl*-MDMA (supplied by the National Institute on Drug Abuse, Rockville, MD) dissolved in 0.9% saline (5 or 10 mg/kg as specified, calculated as the free base) after the indicated time interval after each pretreatment. Rats were sacrificed by decapitation 3 hr after MDMA, and the brain removed immediately and placed on an iced glass plate. The frontal cortex, hippocampi and neostriata (caudate putamen and globus pallidus) were removed bilaterally, frozen on dry ice and stored at −80°C until assayed.

Longer-term experiments. In experiments designed to assess the role of DA in the longer-term serotonergic effects of MDMA, rats were subjected to a multiple dosing (subacute) regimen of concurrent MDMA and AMT. In this experiment, MDMA (0, 2.5, 5 or 10 mg/kg; expressed as the free base) was dissolved in saline and administered s.c. to rats once every 6 hr, for a total of 5 doses. AMT was administered i.p. (60 mg/kg per dose) concurrent with each MDMA dose. Control rats received concurrent injections of saline vehicle. Rats were sacrificed 18 hr after the final dose and the neostriata were removed for assay as described above.

In a final set of experiments, rats were pretreated with either AMT (120 mg/kg; 90 min before MDMA), reserpine (5 mg/kg; 12 hr before MDMA), GBR 12909 (20 mg/kg, expressed as the free base, 15 min before MDMA; the compound was dissolved in propylene glycol and water, 3:1) or the respective vehicle. GBR 12909 was generously provided by NOVO Industri A/S (Copenhagen, Denmark). Pretreatment was followed by a single s.c. injection of MDMA (20 mg/kg, expressed as the free base) or saline (control). Rats were sacrificed 3 days after MDMA and the specific brain regions removed as described previously.

TPH assay. Individual brain tissues were weighed and homogenized in 140 μl of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Sigma Chemical Co.), pH 7.4, containing 0.2% Triton X-100 and 5 mM dithiothreitol. After centrifugation at 27000 × *g* for 15 min,

duplicate 7.5-μl aliquots of supernatant were removed and assayed for TPH activity by a modified $^{14}CO_2$-trapping procedure (Ichiyama *et al.*, 1970; Sitaram and Lees, 1978). The assay was run under V_{max} conditions using *dl*-6-methyl-5,6,7,8-tetrahydrobiopterin (Sigma Chemical Co.) as cofactor, and is described in detail elsewhere (Hotchkiss *et al.*, 1979).

Assay for monoamines and their major metabolites. Neostriatal tissues contralateral to those used in the determination of TPH activity (with the exception of tissues from 6-OHDA-pretreated animals) were assayed for monoamine and monoamine metabolite content by high-performance liquid chromatography. Individual neostriata were weighed and homogenized in 500 μl mobile phase buffer (0.15 M monochloroacetic acid, 2.0 mM EDTA, 0.1 mM *l*-octanesulfonic acid and 12.5% methanol; pH 2.9) and centrifuged at 4800 × *g* for 30 min. The supernatant fraction was subsequently filtered through a 0.2-μm microfilter system (Bioanalytical Systems, Inc., West Lafayette, IN) and 50-μl aliquots were injected onto an 12.5-cm Partisphere C_{18} reversed-phase column equipped with a 1-cm R.P. guard column (Whatman Inc., Clifton, NJ). The eluent was monitored with an amperometric electrochemical detector (model LC-4B; Bioanalytical Systems), with the potential set at +0.73 V. Tissue levels of 5-HT, 5-HIAA and DA were quantitated by comparison with standards of known concentration prepared in mobile-phase buffer and calculated as micrograms per gram of tissue.

Statistics. Results are expressed as the means ± S.E.M. Values of *n* represent the number of animals or, in the 6-OHDA experiments, the number of individual neostriata, and were ≥5 in all experiments. Statistical evaluation of multigroup data was performed by a two-way analysis of variance followed by the Newman-Keuls test for multiple comparisons. Statistical analysis of two-sample data (comparison of mean percentages) was performed by the two-tailed Student's *t* test. Statistical significance between groups was defined as $P < .05$.

Results

Figure 1 shows the effect of prior central DA depletion on the immediate (3 hr) decline in striatal TPH activity caused by a single 5-mg/kg dose of MDMA. At the time of sacrifice, inhibition of DA synthesis by prior AMT administration (pretreatment 1) had decreased neostriatal DA to 38.6 ± 1.0% of saline control, depletion of DA from central storage sites by prior reserpine injection (pretreatment 2) decreased neostriatal DA to 3.5 ± 0.3% of control and the combined pretreatment of AMT + reserpine (pretreatment 3) decreased neostriatal DA to 0.7 ± 0.1% of control. Because rats were sacrificed 3 hr after MDMA, striatal DA depletion at the time of MDMA administration may have differed slightly from the values listed. All three pretreatments significantly attenuated the 3-hr MDMA-induced loss of enzyme activity; however, the interpretation of results was complicated by the fact that the reserpine and the combined AMT + reserpine pretreatments alone significantly elevated TPH activity as compared to vehicle-injected controls (fig. 1). In these instances, analysis of results was approached in an alternate manner: individual TPH activity values from MDMA-treated rats were converted to a percentage of their respective (same pretreatment) saline-treated control mean; statistical analyses were subsequently performed on these percentage values. Analyzed in this way, both reserpine pretreatments significantly blocked the MDMA-induced decline in TPH activity (when expressed as the mean percentage ± S.E.M. of their respective controls, enzyme activity values were: 74.6 ± 3.4% for reserpine-MDMA and 71.7 ± 4.2% for reserpine + AMT-MDMA *vs.* 50.3 ± 1.3% for vehicle-MDMA; pretreatment *vs.* vehicle: $P < .01$). Interestingly, the protection afforded by these two pretreatments was similar in magnitude, *i.e.*, the addition of AMT to the pretreatment regimen did not confer any additional protection over reserpine alone (fig. 1).

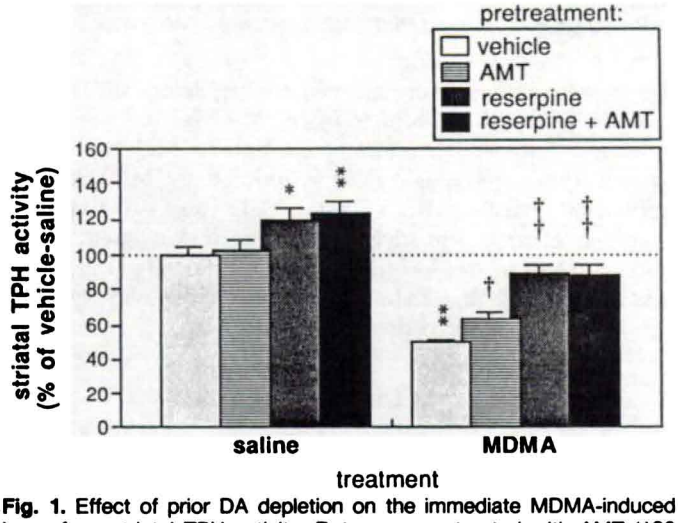

Fig. 1. Effect of prior DA depletion on the immediate MDMA-induced loss of neostriatal TPH activity. Rats were pretreated with AMT (120 mg/kg i.p.), reserpine (5 mg/kg i.p.) or reserpine + AMT (5 and 60 mg/kg, respectively, i.p.) 90 min, 12 hr or 12 hr + 90 min, respectively, before acute MDMA (5 mg/kg s.c.) or saline (control); animals were sacrificed 3 hr later. Results presented are the means ± S.E.M. (n = 6–11), expressed as a percentage of control (vehicle-saline). Control value for TPH activity was 49.2 ± 1.9 nmol/g of tissue per hr. * $P < .05$; ** $P < .01$ *vs.* vehicle-saline, † $P < .05$; ‡ $P < .01$ *vs.* vehicle-MDMA. See "Results" for discussion of statistical significance.

Figure 2 shows the effect of prior 6-OHDA-induced nigral lesions on the immediate (3 hr) MDMA (5 or 10 mg/kg)-induced decreases in regional TPH activity. Neostriatal TH activity was assessed bilaterally as an indicator of individual neostriatal dopaminergic denervation. Of the striatal tissues included in the calculations for figure 2 (*i.e.*, those with a > 80% TH depletion), mean TH activity in lesioned striata was 9.4 ± 0.6% of tissues from sham-lesioned rats (mean TH activity from the sham-saline group was 2546.4 ± 111.3 nmol/g of tissue per hr). Examination of figure 2 reveals that the nigral 6-OHDA pretreatment, alone, induced a significant increase in TPH activity, in both the striatum and hippocampus. When values from MDMA-treated rats were expressed as a percentage ± S.E.M. of their respective saline-treated control mean (see above), neostriatal tissues from 6-OHDA-lesioned rats were significantly protected from the TPH-depleting effects of either a 5-mg/kg dose of MDMA (TPH activity for the 6-OHDA-MDMA group was: 75.3 ± 3.5% of control *vs.* 61.9 ± 3.2% of control for sham-MDMA, $P < .01$) or a 10-mg/kg dose of MDMA (67.6 ± 5.1% for 6-OHDA-MDMA *vs.* 37.5 ± 2.3% for sham-MDMA, $P < .01$). The frontal cortex and hippocampus, however, were not protected by prior nigral lesions (when values were expressed as percentages of respective controls, no significant differences were found between the sham-MDMA and 6-OHDA-MDMA groups in either region, data not shown).

Figures 3 and 4 represent the results from experiments designed to assess the protective effect of AMT pretreatment against longer-term MDMA-induced serotonergic toxicity. "Toxic" serotonergic damage was induced by either a multiple dosing regimen of MDMA (five doses over a 24-hr period), or a high single dose of MDMA (20 mg/kg); these treatments cause persistent decreases in central serotonergic parameters for up to 1 week after treatment (Schmidt, 1987b; Stone *et al.*, 1987). Figure 3 depicts the residual serotonergic effects of multiple doses of MDMA (2.5, 5 or 10 mg/kg; with or without

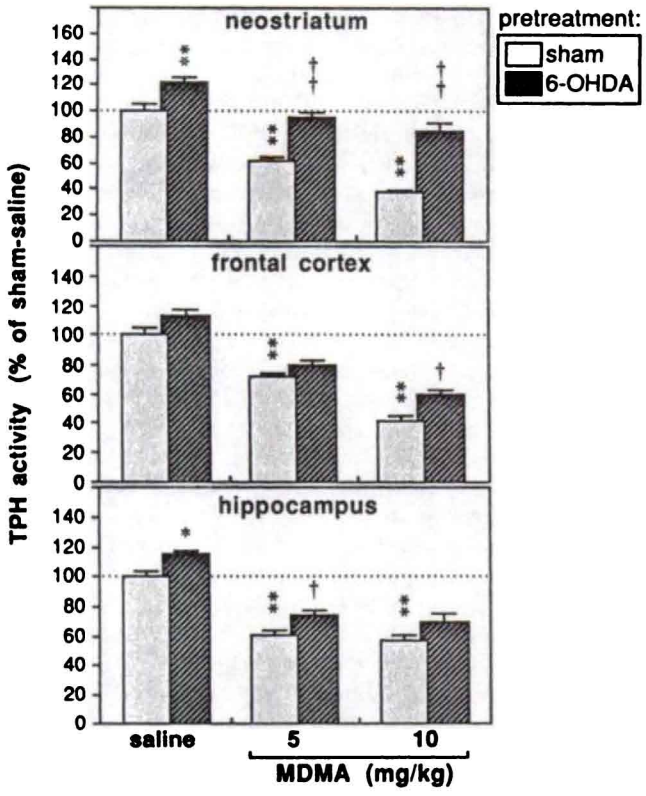

Fig. 2. Effect of prior substantia nigral lesions on the immediate MDMA-induced decreases in regional TPH activity. Lesions were induced bilaterally by local injection of 4 μg of 6-OHDA-8 μl 0.1% ascorbate saline-side. Control rats received sham lesions of ascorbate vehicle alone. After a 7- to 10-day recovery period, acute MDMA (5 or 10 mg/kg) was administered s.c. and rats were sacrificed 3 hr later. Results are the means ± S.E.M., expressed as a percentage of sham-saline (n = 22 for sham-saline group, n = 14 for 6-OHDA-saline group, n = 6–12 for MDMA-treated groups). Control TPH activities (in nanomoles per gram of tissue per hour) were: striatum, 42.2 ± 2.3; frontal cortex, 77.3 ± 3.7; hippocampus, 52.2 ± 1.8. * $P < .05$; ** $P < .01$ *vs.* sham-saline, † $P < .05$, ‡ $P < .01$ *vs.* corresponding sham-MDMA. See "Results" for discussion of statistical significance.

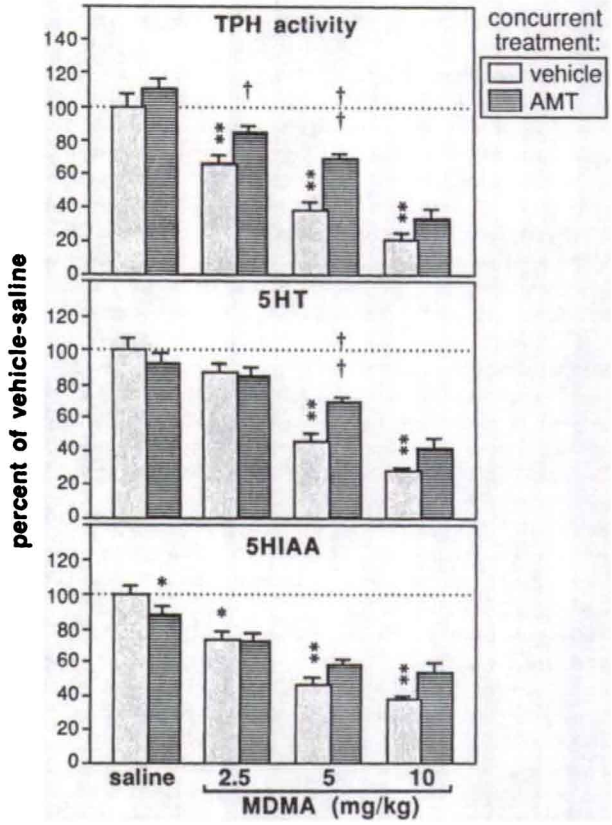

Fig. 3. Effect of concurrent AMT on the toxic serotonergic deficits induced by subacute MDMA. Rats were administered multiple doses of MDMA (0, 2.5, 5 or 10 mg/kg s.c.; 5 doses, 1 every 6 hr). Concurrent with each MDMA dose, AMT (60 mg/kg) or saline vehicle was administered i.p. Rats were sacrificed 18 hr after the last dose. Data are represented as the means ± S.E.M. (n = 17 for vehicle-saline and AMT-saline groups, n = 6–8 for other groups) and are expressed as a percentage of control (vehicle-saline). Control values were: TPH activity (in nanomoles per gram of tissue per hour), 43.3 ± 3.2; 5-HT and 5-HIAA (in micrograms per gram of tissue), 0.433 ± 0.024 and 0.509 ± 0.026, respectively. * $P < .05$; ** $P < .01$ *vs.* corresponding vehicle-saline group, † $P < .05$, ‡ $P < .01$ *vs.* corresponding vehicle-MDMA group.

concurrent AMT treatment), 18 hr after the last dose. In this dosing regimen, AMT exhibited significant neuronal protection primarily against the 5-mg/kg MDMA dose (fig. 3), although the protection afforded by AMT against the depletion of 5-hydroxyindoles induced by 10 mg/kg of MDMA was statistically significant when values were analyzed as a percentage of their respective treatment control mean (5-HT values, expressed as the mean percentage ± S.E.M. of their respective controls were: 46.0 ± 6.0% for AMT-MDMA *vs.* 28.0 ± 2.2% for vehicle-MDMA, $P < .05$; 5-HIAA values were: 61.6 ± 7.7% for AMT-MDMA *vs.* 38.0 ± 2.6% for vehicle-MDMA, $P < .05$).

The residual serotonergic effects remaining 3 days after a single high dose of MDMA (20 mg/kg) were almost completely prevented by AMT pretreatment (fig. 4). Whereas striatal TPH activity from vehicle-pretreated rats was depressed to 46 ± 3% of control at this time ($P < .01$ *vs.* saline), striatal TPH activity from those rats pretreated with AMT was not statistically different from control activity (84 ± 7%). Protective effects of AMT were also observed in the hippocampus (MDMA alone decreased TPH activity to 51 ± 6% of control *vs.* 80 ± 6% of control for the AMT-MDMA group, $P < .01$) and the frontal cortex (61 ± 2% for MDMA alone *vs.* 86 ± 6% for AMT-MDMA, $P < .01$; data not shown). Similar to AMT, reserpine pretreatment completely blocked the persistent depletion of striatal TPH activity induced by MDMA (fig. 5).

Figure 6 shows the effect of DA-uptake inhibition on the longer-term serotonergic deficits induced by MDMA. Prior treatment with GBR 12909 (1-[2-[bis-(4-fluorophenyl) methoxy]ethyl-4-(3-phenylpropyl)piperazine), a potent and selective DA-uptake blocker, significantly attenuated the persistent decrease in both striatal TPH activity and 5-hydroxyindoles caused by a high single dose of MDMA.

Discussion

The results of this study suggest that endogenous DA is involved in both the immediate and the longer-term serotonergic effects of MDMA. Depletion of brain catecholamines with either AMT or reserpine partially blocked the initial (3 hr) decline in TPH enzyme activity normally observed after MDMA (fig. 1). Moreover, this protective effect of catecholamine depletion could be confined selectively to a specific brain region, the neostriatum, by destruction of the striatal dopaminergic afferents with 6-OHDA-induced substantia nigral lesions. Because dopaminergic input to the frontal cortex or

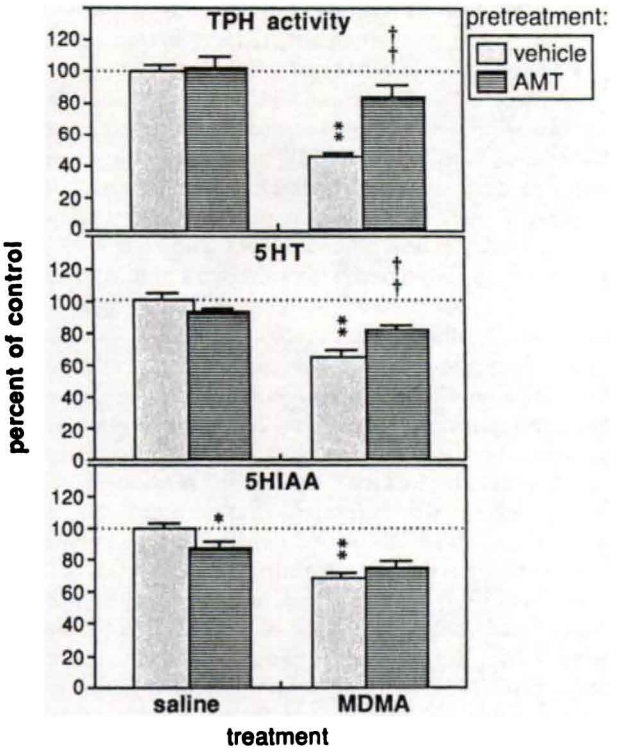

Fig. 4. Effect of AMT pretreatment on the toxic serotonergic deficits induced by acute MDMA. AMT (120 mg/kg) or saline vehicle was administered i.p. 90 min before a single dose of MDMA (20 mg/kg s.c.); rats were sacrificed 3 days later. Results are represented as the means ± S.E.M. (n = 7), expressed as a percentage of control (vehicle-saline). Control values were: TPH activity (in nanomoles per gram of tissue per hour), 50.7 ± 2.4; 5-HT and 5-HIAA (in micrograms per gram of tissue), 0.538 ± 0.022 and 0.368 ± 0.011, respectively. * P < .05; ** P < .01 *vs.* vehicle-saline; ‡ P < .01 *vs.* vehicle-MDMA.

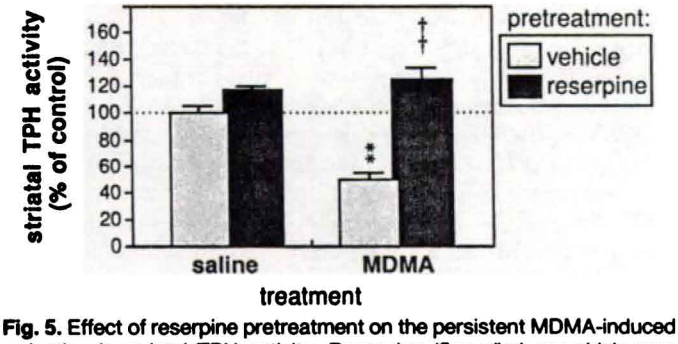

Fig. 5. Effect of reserpine pretreatment on the persistent MDMA-induced reduction in striatal TPH activity. Reserpine (5 mg/kg) or vehicle was administered i.p. 12 hr before a single dose of MDMA (20 mg/kg s.c.) or saline; rats were sacrificed 3 days later. Results are represented as the means ± S.E.M. (n = 6–7), expressed as a percentage of control (vehicle-saline). Control TPH activity was 43.4 ± 2.6 nmol/g of tissue per hr. ** P < .01 *vs.* vehicle-saline; ‡ P < .01 *vs.* vehicle-MDMA.

hippocampus was not altered, these regions were not protected against MDMA-induced TPH depletion (fig. 2).

Surprisingly, reserpine pretreatment afforded a greater protection against the immediate MDMA-induced decline of TPH activity (fig. 1) than did prior AMT. Although reserpine caused a greater degree of DA depletion than did AMT (90% *vs.* 61% depletion), the two drugs are believed to act on different DA pools: whereas reserpine depletes monoamines primarily from vesicular stores by irreversibly damaging vesicular membranes (Dählstrom *et al.*, 1965), AMT depletes the newly-synthesized neurotransmitter pool. It is this latter pool of DA from which

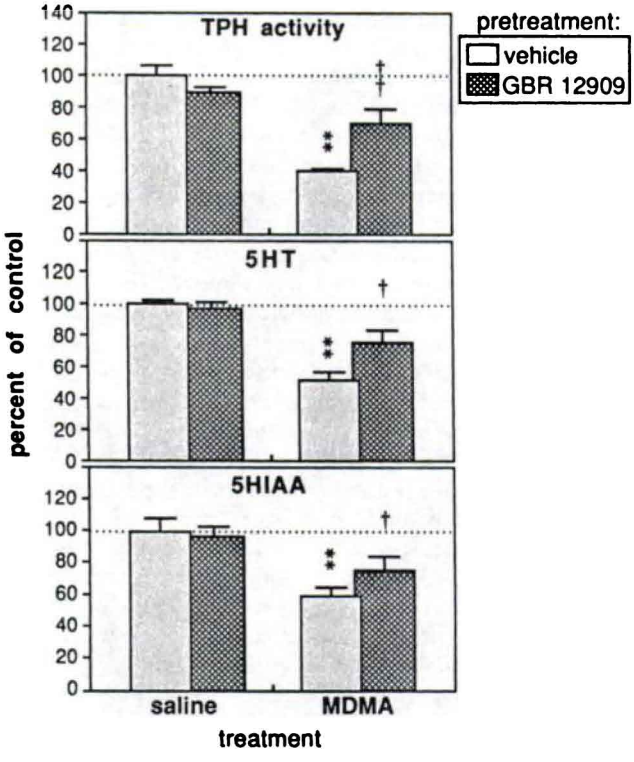

Fig. 6. Effect of DA-uptake inhibition on the toxic serotonergic deficits induced by acute MDMA. GBT 12909 (20 mg/kg i.p.) or vehicle was administered 15 min before a single dose of MDMA (20 mg/kg s.c.); rats were sacrificed 3 days later. Results depicted are the means ± S.E.M. (n = 5–6), expressed as a percentage of control (vehicle-saline). Control values were: TPH activity (in nanomoles per gram of tissue per hour), 53.7 ± 3.1; 5-HT and 5-HIAA (in micrograms per gram of tissue), 0.553 ± 0.013 and 0.502 ± 0.040, respectively. ** P < .01 *vs.* vehicle-saline; † P < .05; ‡ P < .01 *vs.* vehicle-MDMA.

amphetamine and its congeners are thought to cause release, most likely *via* a carrier-mediated "exchange-diffusion" process (Raiteri *et al.*, 1979; Fischer and Cho, 1979). Thus, whereas inhibition of catecholamine synthesis with AMT greatly attenuates amphetamine-induced DA release (Parker and Cubeddu, 1986b; Butcher *et al.*, 1988), reserpine pretreatment reportedly does not alter the amount of DA released by amphetamine (Niddam *et al.*, 1985; Butcher *et al.*, 1988), as catecholamine synthesis is maintained. The effects of prior reserpine are not entirely clear, however, as some investigators have reported a significant enhancement of amphetamine-induced DA release (Ross and Renyi, 1978), whereas others found the amount of DA released by amphetamine to be significantly reduced in reserpinized tissues (Parker and Cubeddu, 1986a,b). Inasmuch as MDMA induces DA release in a manner similar to amphetamine (*i.e.*, both are Ca^{++}-independent and carrier-mediated; Schmidt *et al.*, 1987), one would expect depletion of newly synthesized "preferentially released" DA to afford a greater protection than depletion of storage DA against DA-release-mediated effects of MDMA. However, inasmuch as reserpine depletes not only DA but other monoamines as well, and because reserpine pretreatment alone significantly elevated TPH activity (fig. 1), some factor(s) other than DA depletion might be involved in reserpine-mediated neuronal protection.

Reserpine pretreatment does not block the central behavioral effects of amphetamine, as does AMT, but may actually enhance amphetamine-induced stereotypy (Morpurgo and Theobald, 1966; Randrup and Jonas, 1967; Scheel-Krüger, 1971) and

locomotion (Stolk and Rech, 1967), although these behaviors occur sooner after drug administration and are of shorter duration. In the present investigation, whereas reserpine pretreatment did not diminish the initial behavioral responses to MDMA (enhanced locomotion, grooming and rearing), it did decrease the duration of drug-induced behavior (by 2 hr after MDMA, reserpine-pretreated rats were calm or asleep whereas nonreserpinized rats still exhibited drug-induced excitatory and locomotor behaviors; unpublished observations). Assuming behavior reflects ongoing neurochemical events, these observations suggested that reserpine pretreatment decreased the duration of the neurochemical response to MDMA *in vivo*.

The immediate serotonergic deficits caused by MDMA are reversible as evidenced by the ability of 5-HT-uptake inhibitors to block the development of long-term toxicity, even when administered 3 to 6 hr after MDMA (Schmidt, 1987b; Schmidt and Taylor, 1987) at a time when serotonergic parameters are already depressed maximally; moreover, striated TPH activity, which was reduced profoundly (to < 60% of control) 4 hr after a single low dose of MDMA (5 mg/kg), had returned to control values by 24 hr after drug administration (92% of control, unpublished observations) yet when the same dose was administered repeatedly at 6-hr intervals (five doses total), enzyme activity remained depressed for up to 30 days after treatment (Stone *et al.*, 1987). These observations suggest there is a critical time period during which the drug, drug metabolite, or endogenous neurochemical must remain in contact with neuronal circuitry to result in long-term effects. If these initial drug-mediated events are halted abruptly, as occurs with the subsequent administration of the 5-HT-uptake inhibitor fluoxetine, the development of toxicity likewise ceases and the brain is able to recover rapidly. Viewed in this context, the ability of reserpine pretreatment to prevent MDMA-induced serotonergic deficits may be due not to a blockade of MDMA-induced DA release, release which probably still occurs, at least initially, as evidenced by drug-induced behavior, but to the fact that the neurochemical response does not persist for the critical period of time necessary to induce toxicity, as reflected in the loss of drug-induced behavior in reserpinized rats 2 hr after MDMA (see above).

The protective effects of prior AMT or reserpine, reported herein, against the immediate MDMA-induced decline of TPH activity, are in contrast to a recent study by Schmidt and Taylor (1987) who found no protective effect of these two agents. However, these investigators used a higher dose of MDMA (10 *vs.* 5 mg/kg in the present study), the neurochemical effects of which may be sufficiently potent to overwhelm the initial neuronal protection afforded by partial DA depletion.

MDMA and its analogs cause pronounced release of 5-HT from rat hippocampal and striatal slices *in vitro* (Johnson *et al.*, 1986; Schmidt *et al.*, 1987; Schmidt, 1987a); this initial action of MDMA *in vivo*, in conjunction with drug-induced blockade of 5-HT reuptake (Steele *et al.*, 1987) and loss of 5-HT synthesizing capacity (Stone *et al.*, 1986; Schmidt and Taylor, 1987), probably accounts for the rapid decrease in 5-HT levels observed after MDMA. Schmidt and Taylor (1987) have suggested a role for drug-released 5-HT in the initial MDMA-induced reduction of enzyme activity, presumably through autoreceptor feedback mechanisms as coadministration of the autoreceptor antagonist, methiothepin, partially antagonized the immediate serotonergic effects of MDMA (*ibid.*). Whereas the ability of reserpine to reduce intraneuronal stores of 5-HT may at least partially explain its initial neuroprotective effects, reserpine-mediated protection against the long-term serotonergic effects of MDMA (fig. 5) most likely does not involve 5-HT mechanisms. This conclusion is based on the following two lines of evidence: first, as discussed previously by Johnson *et al.* (1987a), the ability of amphetamine analogs to induce long-term toxicity appears to correlate more directly with their DA-releasing potency than their 5-HT-releasing potency. Thus, whereas MDA and both its N-methylated and N-ethylated derivatives (MDMA and MDE, respectively) are of similar potency in inducing 5-HT release *in vitro* (Johnson *et al.*, 1986; Schmidt, 1987a), the DA-releasing potency of these compounds varies according to structure: an increase in the size of the N-alkyl substituent correlates with a decrease in the compound's ability to elicit DA release; correspondingly, those compounds with a lower DA-releasing potency also exhibit reduced long-term serotonergic effects (Schmidt, 1987a). Also of interest in this regard, the "*S*"-(+)-stereoisomer of either MDA or MDMA, that isomer with the greater monoamine-releasing ability (Johnson *et al.*, 1986; Schmidt *et al.*, 1987) and the greater inhibitory effect on striatal synaptosomal DA uptake (Steele *et al.*, 1987), is more potent at causing long-term toxicity than its "*R*"-(−)-counterpart (Schmidt *et al.*, 1987; Schmidt, 1987b). Second, inhibition of 5-HT synthesis with *p*-chlorophenylalanine, a competitive inhibitor of TPH, does not protect against the long-term serotonergic deficits caused by other amphetamine analogs such as METH (Schmidt and Gibb, 1983) or PCA (Fuller *et al.*, 1975).

Results from the present investigation suggest endogenous DA may be only partially involved in the immediate MDMA-induced loss of TPH activity, as prior DA depletion never completely prevented this immediate effect, even when striatal DA was reduced to <1% of control after combined AMT + reserpine pretreatment (fig. 1). Endogenous DA appears to play an integral role, however, in the development of long-term toxicity. Not only did prior AMT (fig. 4) or reserpine (fig. 5) completely prevent the persistent (3 days) reduction in TPH activity induced by a high single dose of MDMA, but specific blockade of the DA-uptake carrier with GBR 12909 (van der Zee *et al.*, 1980; Heikkila and Manzino, 1984) greatly attenuated this long-term loss of enzyme activity (fig. 6). Because, *in vitro*, GBR 12909 is 50- or 150-fold more potent at inhibiting the uptake of DA than either norepinephrine or 5-HT, respectively (van der Zee *et al.*, 1980), and, at the concentration used in the present study, GBR 12909 would most likely exert no appreciable effect on either norepinephrine (Heikkila and Manzino, 1984) or 5-HT uptake mechanisms, the neuronal protection afforded by this compound can be attributed to its presumed inhibition of carrier-mediated (MDMA-induced) DA release *in vivo*, an inhibitory action characteristic of DA-uptake blockers in general (Raiteri *et al.*, 1979; Fischer and Cho, 1979). The protective effect of DA-uptake blockade reported here lends additional credence to the idea that drug-released DA plays an important role in the development of MDMA-induced toxicity.

DA and other catecholamines have long been recognized as potential cytotoxic agents. Not only is DA itself directly toxic to rat brain cells (as measured by the inhibition of creatinine and adenylate kinases; Maker *et al.*, 1986) and to mammalian cells, both *in vitro* and *in vivo* (Prasad and Kolmorgen, 1969), but monoamine oxidase-catalyzed brain metabolism of DA and other biogenic amines can liberate H_2O_2 (Sinet *et al.*, 1980), a cytotoxic compound which, under normal conditions, is rapidly

inactivated by protective brain enzymes such as catalase or glutathione peroxidase. Under oxidizing conditions *in vitro*, DA is nonenzymatically oxidized more rapidly than either norepinephrine or epinephrine (Graham, 1978), resulting in a greater generation of cytotoxic species. Hypothetical auto-oxidation of DA *in vivo* may result not only in the liberation of reactive oxygenating species such as H_2O_2, superoxide radicals and hydroxyl radicals, cytotoxic agents which have been hypothesized to play a role in the pathological neurodegenerative processes associated with Alzheimer's (Sinet *et al.*, 1980) and Parkinson's diseases (Ambani *et al.*, 1975; Perry *et al.*, 1982), and have been implicated as mediators of the neuronal destruction induced by certain monoamine neurotoxins including 6-OHDA and 6,7-dihydroxytryptamine (Cohen and Heikkila, 1974), but may yield highly reactive quinone species capable of binding covalently to nucleophilic cell substituents; indeed, Tse and coworkers (1976) have concluded that, upon formation *in vivo*, interaction of the DA auto-oxidation product DA-*o*-quinone with intracellular particulate sulfhydryl groups (*e.g.*, those present in cell enzymes and neuronal membranes) would predominate over either intracyclization of the quinone to the corresponding aminochrome or rereduction of the quinone to the original catechol by endogenous reductants such as ascorbic acid. Alternatively, DA might undergo nonenzymatic hydroxylation resulting in formation of the neurotoxin 6-OHDA, a process which has been demonstrated *in vitro* (Slivka and Cohen, 1985) and has been hypothesized to account for the trace amounts of 6-OHDA detected in rat striatum after a single large dose of METH (Seiden and Vossmer, 1984). However, the actual *in vivo* formation of this neurotoxin from endogenous DA has been questioned by several investigators (Slivka and Cohen, 1985; Rollema *et al.*, 1986). A role for auto-oxidation products of endogenous DA in the monoaminergic neurotoxic effects of both amphetamine (Fuller and Hemrich-Luecke, 1982) and METH (Schmidt *et al.*, 1985; Seiden and Vossmer, 1984) has been hypothesized previously.

The precise mechanism by which drug-released DA interacts with serotonergic systems to induce toxic damage remains a matter of speculation. Under conditions of enhanced DA release coupled with reduced DA catabolism consequent to monoamine oxidase inhibition (Fellows and Bernheim, 1950), two effects presumed to occur *in vivo* after acute MDMA administration, the resulting high local concentrations of extracellular DA would increase the availability of this transmitter for 5-HT carrier-mediated uptake into neighboring serotonergic nerve terminals (Waldmeier, 1985), a process which occurs readily in model systems *in vitro* and which is blocked effectively by inhibitors of 5-HT uptake (Schmidt and Lovenberg, 1985). In this regard, Schmidt and Lovenberg (1985) have hypothesized that the ability of 5-HT uptake inhibitors to selectively prevent METH-induced serotonergic deficits *in vivo*, stems from a blockade by these drugs of 5-HT carrier-mediated uptake of DA into 5-HT terminals (*ibid*), a mechanism which may apply as well to the inhibition by fluoxetine (Schmidt, 1987b) of MDMA-induced serotonergic deficits. Once inside the serotonergic terminal, the DA might undergo oxidative processes resulting in the generation of cytotoxic metabolites or byproducts, a process which may lead to eventual destruction of the 5-HT terminal. In addition, DA and other catechol compounds are effective inhibitors of TPH enzyme activity (Jequier *et al.*, 1969; Karobath *et al.*, 1972), an action which might play a role in the immediate, reversible effect of MDMA on enzyme function. These hypothetical models do not account for the differential toxicity of MDMA and other amphetamine analogs on dopaminergic systems, however. Undoubtedly, factors in addition to DA-releasing potency influence the specific toxic effects of these compounds; such factors probably include the degree of drug lipophilicity, drug affinity for 5-HT and DA membrane uptake carriers and/or local drug tissue binding, and drug metabolism and half-life in brain and may also involve the inherent metabolic differences which exist between central catecholamine and 5-HT neurons (Hillarp *et al.*, 1966).

The ability of catecholamine depletion to elevate striatal TPH activity was a fairly consistent finding throughout the present study. Not only did reserpine (15 hr before sacrifice, fig. 1) and 6-OHDA (7–10 days before sacrifice, fig. 2) significantly elevate striatal TPH activity, but repeated AMT elicited a similar, although statistically nonsignificant, effect (fig. 3). Reciprocal interactions between 5-HT and catecholamine systems are known to exist in the striatum as well as other brain areas (for review see Samanin and Garattini, 1975). Previous studies have reported an increase in 5-HT synthesis or turnover after depletion of catecholamines induced by either AMT (Zweig and Axelrod, 1969; Stein *et al.*, 1974), 6-OHDA (Blondaux *et al.*, 1973; Lee and Geyer, 1984), or DA β-hydroxylase inhibition (Johnson *et al.*, 1972); additionally, Luthman and co-workers (1987) found a proliferative sprouting of striatal 5-HT nerve terminals 8 to 12 weeks after 6-OHDA-induced DA denervation in neonatal rats. Thus, the elevation of TPH activity reported in the present study appears consistent with earlier findings and probably represents a compensatory serotonergic response to the reduced dopaminergic neurotransmission resulting from experimentally induced catecholamine depletion.

Finally, some mention should be made of the role of DA in the toxic effects of other amphetamine analogs. The protection afforded by either AMT or 6-OHDA against the TPH-depleting effects of MDMA (figs. 1, 2 and 3; this study) appeared to be less dramatic than the protective effects of these agents against corresponding METH-induced deficits (Schmidt *et al.*, 1985; Johnson *et al.*, 1987b). Whether these differences reflect the inherent variability in different experimental conditions, or represent actual quantitative differences in the role of drug-released DA in the serotonergic effects of these two analogs remains unresolved. The possible involvement of DA in the serotonergic toxicity induced by other amphetamine analogs such as PCA merits future investigation, particularly in view of the potent DA-releasing capability of PCA *in vivo* (Sharp *et al.*, 1986). Recently, Steranka and Rhind (1987) reported that systemic administration of *l*-cysteine, a potential source of free sulfhydryl groups subsequent to the synthesis of the detoxicant, glutathione, attenuated the toxic effects of both amphetamine on striatal DA content and of PCA on striatal 5-hydroxyindole concentrations. Although these results do not implicate DA *per se* as the toxic mediator, they do support the notion that the neuronal damage induced by these compounds is at least partially mediated *via* reactive oxygenating species.

In conclusion, endogenous catecholamines appear to be at least partially involved in the immediate serotonergic effects of MDMA as prior depletion of DA with either AMT, reserpine or 6-OHDA significantly attenuated the initial drug-induced reduction in striatal TPH activity. Furthermore, endogenous catecholamines, specifically drug-released DA, appeared to play an integral role in the development of the long-term (toxic)

effects of MDMA, as the persistent MDMA-induced striatal TPH deficit could be prevented completely by prior catecholamine depletion, and significantly attenuated by pretreatment with the potent and selective DA-uptake blocker GBR 12909, a presumed inhibitor of carrier-mediated (drug-induced) DA release. The fact that other amphetamine analogs are potent releasers of DA *in vitro*, and that a number of these compounds are demonstrated neurotoxins *in vivo*, suggests a possible common role for drug-released DA in the central neurotoxicities induced by these agents.

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