

Accumulation of Neurotoxic Thioether Metabolites of 3,4-(±)-Methylenedioxymethamphetamine in Rat Brain

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ABSTRACT

The serotonergic neurotoxicity of 3,4-(±)-methylenedioxymethamphetamine (MDMA) appears dependent upon systemic metabolism because direct injection of MDMA into the brain fails to reproduce the neurotoxicity. MDMA is demethylated to the catechol metabolite *N*-methyl- α -methyl-dopamine (*N*-Me- α -MeDA). Thioether (glutathione and *N*-acetylcysteine) metabolites of *N*-Me- α -MeDA are neurotoxic and are present in rat brain following s.c. injection of MDMA. Because multidose administration of MDMA is typical of drug intake during rave parties, the present study was designed to determine the effects of multiple doses of MDMA on the concentration of neurotoxic thioether metabolites in rat brain. Administration of MDMA (20 mg/kg s.c.) at 12-h intervals for a total of four injections led to a significant accumulation of the *N*-Me- α -MeDA thioether metabolites in striatal dialysate. The area under the curve (AUC)_{0-300 min} for 5-(glutathion-S-yl)-*N*-Me- α -MeDA increased 33% between the first and fourth injections

and essentially doubled for 2,5-bis-(glutathion-S-yl)-*N*-Me- α -MeDA. Likewise, accumulation of the mercapturic acid metabolites was reflected by increases in the AUC_{0-300 min} for both 5-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA (35%) and 2,5-bis-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA (85%), probably because processes for their elimination become saturated. Indeed, the elimination half-life of 5-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA and 2,5-bis-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA increased by 53 and 28%, respectively, between the first and third doses. Finally, although the C_{max} values for the monothioether conjugates were essentially unchanged after each injection, the values increased by 38 and ~50% for 2,5-bis-(glutathion-S-yl)-*N*-Me- α -MeDA and 2,5-bis-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA, respectively, between the first and fourth injections. The data indicate that neurotoxic metabolites of MDMA may accumulate in brain after multiple dosing.

The serotonergic neurotoxicity 3,4-(±)-methylenedioxymethamphetamine (MDMA, Ecstasy) and 3,4-(±)-methylenedioxymethamphetamine (MDA) is dependent on the route of administration (O'Shea et al., 1998). Direct injection of MDMA and MDA into the brain fails to reproduce the acute or long-term neurotoxic effects evident following peripheral administration of these drugs (Schmidt et al., 1987; Paris and Cunningham, 1992), suggesting that systemic (liver) metabolism contributes to the neurotoxicity of MDMA and MDA. Indeed, although MDMA targets the serotonergic system in humans, nonhuman primates, and rats, the finding that MDMA does not affect the serotonergic system in mice but rather targets the dopaminergic system has been attributed to metabolic differences between species (Logan et al., 1988; Escobedo et al., 2005). Perhaps the most convincing evidence that peripheral

metabolism of MDMA is required for neurotoxicity was provided by the work of Esteban et al. (2001). In this study, MDMA was perfused into the hippocampus in amounts sufficient to give rise to the range of concentrations observed following peripheral administration of neurotoxic doses of MDMA. After perfusion, acute monoamine release was observed in the absence of long term depletions in 5-HT levels. These data are consistent with the hypothesis that peripheral generation of neurotoxic metabolites contributes to MDMA-induced serotonergic neurotoxicity.

MDMA is *N*-demethylated to MDA and *O*-demethylated to *N*-methyl- α -methyl-dopamine (*N*-Me- α -MeDA) (Fig. 1) (Lin et al., 1992). *N*-Demethylation in humans is a minor metabolic pathway (~10%), but α -MeDA is a metabolite of both MDMA and MDA. Isoenzymes belonging to the CYP2D subfamily, and the CYP2B or CYP3A1 isoforms, catalyze the low- and high- K_m *O*-demethylation reactions, respectively (de la Torre et al., 2004). The catecholamine metabolites are very unstable and are either conjugated with sulfate/gluc-

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ABBREVIATIONS: MDMA, Ecstasy, 3,4-(±)-methylenedioxymethamphetamine; MDA, 3,4-(±)-methylenedioxymethamphetamine; 5-HT, serotonin; *N*-Me- α -MeDA, *N*-methyl- α -methyl-dopamine; α -MeDA, α -methyl-dopamine; GSH, glutathione; HPLC, high-performance liquid chromatography; CEAS, coulometric electrode array system; MS/MS, tandem mass spectrometry; 5-HIAA, 5-hydroxyindole acetic acid; AUC, area under the curve; DA, dopamine; γ -GT, γ -glutamyltranspeptidase; MRP, multidrug resistance-associated protein; OAT, organic anion transporter.

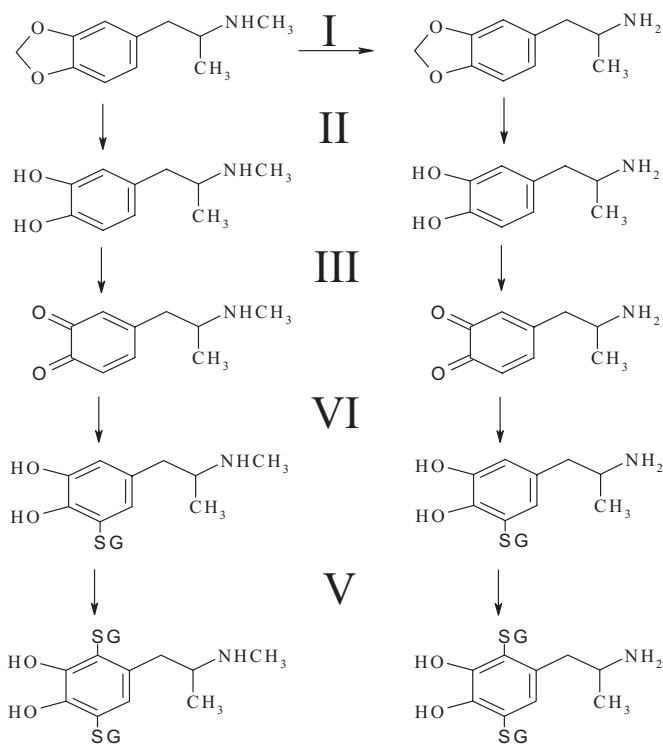


Fig. 1. Metabolism of MDMA and MDA via *O*-demethylation and subsequent oxidation coupled to GSH conjugation. I, MDMA is *N*-demethylated to MDA. II, both MDMA and MDA are *O*-demethylated to *N*-Me- α -MeDA and α -MeDA, respectively. The catecholamine metabolites are very unstable and are either conjugated with sulfate/glucuronic acid or *O*-methylated to 3-*O*-methyl-*N*-Me- α -MeDA or 3-*O*-methyl- α -MeDA in reactions catalyzed by catechol *O*-methyl-transferase. Alternatively, both catechols can rapidly undergo oxidation to the corresponding *ortho*-quinones, which are highly electrophilic (III) and react readily with GSH to form GSH conjugates (IV). V, GSH conjugates can undergo a further round of oxidation to the corresponding quinone-thioethers, followed by addition of a second molecule of GSH. Both the mono- and bis-GSH conjugates are further processed via the mercapturic acid pathway, ultimately yielding the mono- and bis-*N*-acetylcysteine conjugates.

uronic acid or *O*-methylated to 3-*O*-methyl-*N*-Me- α -MeDA or 3-*O*-methyl- α -MeDA in reactions catalyzed by catechol *O*-methyl-transferase. Alternatively, because *N*-Me- α -MeDA and α -MeDA are both catechols, they can rapidly undergo oxidation to the corresponding *ortho*-quinones, which are highly electrophilic, as evidenced by their ability to react readily with the cysteinyl sulfhydryl group in glutathione (GSH) to form GSH conjugates (Hiramatsu et al., 1990; Patel et al., 1991) (Fig. 1). Although conjugation of electrophiles with GSH usually results in detoxication and their subsequent elimination as mercapturic acids, several examples exist where conjugation of GSH with electrophiles results in preservation or enhancement of biological (re)activity (Monks and Lau, 1998). For example, quinone-thioethers retain the ability to redox cycle and produce reactive oxygen species and to arylate tissue macromolecules (Wefers and Sies, 1983; Kleiner et al., 1998a,b). Quinone-thioethers also inhibit enzymes that utilize GSH as a cosubstrate (Monks and Lau, 1997) and, in particular, the 5-*S*-glutathionyl conjugates of dopamine and α -methyl-dopa inhibit human GSH *S*-transferases (Ploemen et al., 1994).

We have been investigating the potential role of thioether metabolites of *N*-Me- α -MeDA and α -MeDA in the neurotoxicity of MDMA and MDA. 5-(Glutathion-*S*-yl)- α -MeDA is me-

tabolized via the mercapturic acid pathway within the central nervous system, forming 5-(cystein-*S*-yl)- α -MeDA and 5-(*N*-acetyl-cystein-*S*-yl)- α -MeDA (Miller et al., 1995). 5-(Glutathion-*S*-yl)- α -MeDA is also readily oxidized to the corresponding quinone-GSH conjugate and undergoes addition of a second molecule of GSH to form 2,5-bis-(glutathion-*S*-yl)- α -MeDA, i.c.v. administration of which causes the selective and prolonged depletion of 5-HT in the striatum, cortex, and hippocampus and neurobehavioral changes consistent with MDA and MDMA (Miller et al., 1997). Moreover, direct injection of 5-(*N*-acetylcystein-*S*-yl)- α -MeDA and 5-(glutathion-*S*-yl)- α -MeDA into the striatum, cortex, and hippocampus produced prolonged depletions in 5-HT and neurobehavioral changes similar to MDA and MDMA (Bai et al., 1999). In particular, 5-(*N*-acetylcystein-*S*-yl)- α -MeDA was an extremely potent serotonergic toxicant. More recently, we have shown that the corresponding mercapturic acid derived from MDMA, 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA, is also a potent serotonergic toxicant (Jones et al., 2005), and, perhaps of greatest significance, we have identified thioether metabolites of *N*-Me- α -MeDA in striatal dialysates of rats administered MDMA peripherally (Jones et al., 2005).

The alarming extensive recreational use of MDMA and MDA and the multitude of adverse effects resulting from their misuse necessitate a complete understanding of their modus operandi. In particular, because hepatic metabolism appears to be a prerequisite for the development of neurotoxicity, a comprehensive understanding of the metabolism and disposition of these amphetamine derivatives seem to be a prerequisite to understanding their neurotoxicology. Therefore, it would seem prudent to understand the metabolism of MDMA and the properties of neurotoxic metabolites to determine the extent to which the neuropharmacology and neurotoxicology of MDMA are coupled.

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX) weighing ~300 g were group-housed and maintained on 12-h light/dark cycle. Food and water were provided ad libitum. Rats were handled for 2 weeks before surgical cannula implantation to reduce stress. Procedures were carried out in accordance with the University of Arizona Institutional Animal Care and Use Committee.

Synthesis and Purification of 5-(Glutathion-*S*-yl)-*N*-methyl- α -MeDA and 2,5-bis(Glutathion-*S*-yl)-*N*-methyl- α -MeDA. The GSH conjugates were synthesized following a procedure similar to that established in our laboratory (Miller et al., 1996; Jones et al., 2005) with modifications. In short, a mixture of 25 mg of *N*-Me- α -MeDA (Southwest Environmental Health Sciences Center Synthetic Chemistry Core Facility, University of Arizona, Tucson, AZ), 100 mg of GSH, and 3 mg of mushroom tyrosinase (1530 units/mg; Sigma-Aldrich, St. Louis, MO) was stirred at 37°C for 30 min in 50 ml of sodium phosphate buffer (50 mM, pH 7.4). The solution was frozen at -80°C and lyophilized to dryness. The resulting dried product was further purified by HPLC (SCL-10A; Shimadzu, Columbia, MD) by dissolving it in 2 ml of 1% formic acid and injecting 100- μ l aliquots onto a Beckman Ultrasphere ODS-5 reverse-phase semipreparative column (25 \times 1-cm i.d.; 5- μ m particle size; Beckman Coulter, Fullerton, CA). The product was eluted using methanol and 1% acetic acid (15:85, v/v) at a flow rate of 2 ml/min, and the eluate was monitored at 280 nm. Fractions of interest were combined and lyophilized to dryness. The resulting product was then analyzed by HPLC-coulometric electrode array system (CEAS) to confirm purity.

To confirm structure, HPLC coupled to tandem mass spectrometry (MS/MS) revealed a single compound with a parent ion corresponding to 5-(glutathion-S-yl)-*N*-Me- α -MeDA (m/z 487). Fragmentation of the 487 MH⁺ ion showed several daughter ions, including GSH (m/z 307.8) and *N*-Me- α -MeDA (m/z 182.9). 2,5-bis-(Glutathion-S-yl)-*N*-Me- α -MeDA was synthesized by dissolving ~100 mg of 5-(glutathion-S-yl)-*N*-Me- α -MeDA in 100 ml of 10% formic acid with 50 mg of sodium periodate. The color changed from light golden to deep purple. GSH was added in excess, and the reaction was incubated overnight. The solution was purified by HPLC as described above. The mobile phase was methanol, and water was adjusted to pH 3 with formic acid (15:85, v/v) at a flow rate of 2 ml/min. The eluate was monitored at 280 nm, collected, and lyophilized to dryness. The product when reanalyzed by HPLC-CEAS gave rise to a single peak, and HPLC-MS/MS confirmed a single ion of MH⁺ 792, corresponding to 2,5-bis-(glutathion-S-yl)-*N*-Me- α -MeDA.

Synthesis and Purification of 5-(*N*-Acetylcystein-S-yl)-*N*-methyl- α -MeDA and 2,5-bis(*N*-Acetylcystein-S-yl)-*N*-methyl- α -MeDA. Mercapturic acid conjugates of *N*-Me- α -MeDA were synthesized following a similar procedure to that described by Jones et al. (2005), with minor modifications. In brief, a mixture of 25 mg of *N*-Me- α -MeDA, 100 mg of *N*-acetylcysteine, and 3 mg of mushroom tyrosinase (1530 units/mg, Sigma-Aldrich) were stirred at 28°C for 30 min in 50 ml of sodium phosphate buffer (50 mM, pH 7.4). The solution was quenched with 2 ml of 88% formic acid, frozen at -80°C and lyophilized to dryness. The dried product was further purified by HPLC (Shimadzu, SCL-10A) by dissolving it in 2 ml of 1% formic acid and injecting 100- μ l aliquots onto a Beckman Ultrasphere ODS-5 reverse-phase semipreparative column (25 \times 1-cm i.d.; 5- μ m particle size). Samples were eluted using methanol and 1% acetic acid (10:85, v/v) at a flow rate of 2 ml/min, and the eluate was monitored at 225 nm. 2,5-Bis-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA was synthesized by dissolving ~100 mg of 5-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA in 100 ml of 10% formic acid with 50 mg of sodium periodate. *N*-Acetylcysteine (50 mg) was then added, and the reaction was incubated overnight. After lyophilization, the reaction mixture was purified by HPLC by dissolving in 2 ml of 1% formic acid and injecting 100- μ l aliquots onto a Beckman Ultrasphere ODS-5 reverse-phase semipreparative column. The mobile phase was methanol and 1% acetic acid (15:85, v/v) at a flow rate of 2 ml/min. The eluate was monitored at 225 nm, collected, and lyophilized to dryness. The product, when reanalyzed by HPLC-CEAS, gave rise to a single peak, and HPLC-MS/MS revealed a single parent ion at MH⁺ 504, corresponding to 2,5-bis-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA.

In Vitro Probe Recovery Calibration and Analysis. Microdialysis probes were constructed in-house as described previously (Duvauchelle et al., 2000; Jones et al., 2005). In short, to determine microdialysis probe recovery calibration, probes were placed in Ringer's solution supplemented with 5-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA, 2,5-bis-(*N*-acetylcystein-S-yl)-*N*-Me- α -MeDA, 5-(glutathion-S-yl)-*N*-Me- α -MeDA, and 2,5-bis-(glutathion-S-yl)-*N*-Me- α -MeDA (20 nM) at 37°C. Hamilton syringes were filled with filtered and freshly prepared Ringer's solution and pumped through the microdialysis probe at a rate of 1.65 μ l/min. Dialysate were collected every 30 min from each probe and assayed by HPLC-CEAS. Microdialysis probe recovery was calculated by comparing the peak heights of each dialysate and those obtained from a standard solution.

Surgical Cannula and Microdialysis Probe Implantation. Rats were anesthetized with 4 ml/kg of a mixture containing chloral hydrate (37.5 mg/ml) and sodium pentobarbital (9.4 mg/ml). A unilateral guide cannula (Plastics One, Roanoke, VA) was stereotaxically implanted above the striatum. Cannulae were positioned following coordinates from bregma (anteroposterior, 0.2 mm; mediolateral, \pm 3.0 mm; dorsoventral, 2.5 mm) (Paxinos and Watson, 1997). A dummy cannula (Plastics One) was inserted in the guide cannula. Rats were housed singly and allowed to recover for 6 to 7 days before further procedures were performed. Microdialysis probes

were lowered through the guide cannula while rats were anesthetized with isoflurane, 12 h before the experiment. Animals already implanted with the microdialysis probes remained overnight with the Ringer's solution pumped through the probe at a rate of 0.2 μ l/min. The syringe pump (Razel, Stamford, CT) speed was increased to 1.63 μ l/min 1 h before the collection of the first baseline sample. Dialysate samples were collected into 0.5-ml tubes, with 0.5 N perchloric acid, at 30-min intervals before (baseline samples) administration of either saline solution (0.9% NaCl) or (\pm)-MDMA (Sigma-Aldrich) (20 mg/kg) and every 30 min for 5 h following each of the four (\pm)-MDMA s.c. injections (12-h intervals). Doses of (\pm)-MDMA capable of producing neurotoxicity in rodents are usually higher than those required to produce similar toxicity in primates. Such a relationship is anticipated by the principles of interspecies scaling, which predicts a single dose of 1.28 mg/kg in humans (~96 mg/75 kg) to be equivalent to a known neurotoxic dose of 20 mg/kg in rats (Ricaurte et al., 2000). Recreational users of (\pm)-MDMA typically use single doses of 75 to 125 mg (\pm)-MDMA, a dose that lies well within the neurotoxic range predicted by interspecies scaling (Ricaurte et al., 2000). Moreover, users of the drug usually ingest multiple doses during the course of a "rave" or at similar recreational gatherings.

HPLC-CEAS Analysis of *N*-Me- α -MeDA Thioether Metabolites and Brain Monoamine Concentrations. *N*-Me- α -MeDA thioether metabolites, 5-HT, and 5-HIAA concentrations were quantified by HPLC coupled to a four-channel coulometric electrode array system (ESA Inc., Chelmsford, MA), using an ESA HR-80 column (8-cm \times 4.6-mm i.d., 3-mm particle size). The potentials applied were set to +0, +150, +300, and +350 mV. The mobile phase consisted of 8 mM ammonium acetate, 4 mM citrate, 54 μ M EDTA, 230 μ M 1-octanesulfonic acid, and 10% methanol, pH 2.5, at a flow rate of 1 ml/min. Quantitation of the *N*-Me- α -MeDA thioether metabolites was determined by comparing the peak area with standards analyzed on the day of dialysate collection. The concentrations of monoamines in brain tissue were determined by HPLC-CEAS following dissection of the brain 7 days after the last injection of MDMA (Bai et al., 1999; Jones et al., 2005). Quantitation of monoamines was determined by comparison of peak areas of samples with standard curves generated from authenticated standards analyzed during each series of sample analyses.

HPLC-MS/MS Detection. Standards were analyzed via HPLC using an HP/Agilent 1050 pumping system (Hewlett Packard/Agilent Technologies, Palo Alto, CA) equipped with a SPHERISORB ODS-2, 5 μ m, 4.6 \times 150-mm column (Alltech Associates, Deerfield, IL), onto which 10 μ l of sample was injected via an HP 1050 autosampler. The isocratic mobile phase consisted of acetic acid/methanol/water (1:14:84, v/v) delivered at a flow rate of 0.5 ml/min for 50 min. UV detection was performed at 254 nm using an HP 1050 Variable Wavelength Detector (Hewlett Packard/Agilent). LC separation was coupled to MS analysis using a Finnigan MAT TSQ 7000 triple quadrupole mass spectrometer (ThermoElectron, San Jose, CA). Analytes were ionized using electrospray ionization at an electrospray ionization source spray voltage of +4.5 kV. Ions were introduced into the mass spectrometer through a heated metal capillary maintained at 250°C.

Pharmacokinetic Analyses. Data were analyzed using the non-compartmental analysis of pharmacokinetics parameters (PK Functions for Microsoft Excel; Joel L. Usansky, Ph.D., Atul Desai, M.S., and Diane Tang-Liu, Ph.D., Department of Pharmacokinetics and Drug Metabolism, Allergan, Irvine, CA; <http://www.boomer.org/pkin/soft.html>). Parameters used for each animal, the mean and S.D., were determined separately.

Statistics. Results from the microdialysis (metabolites and monoamines) analyses are presented as absolute values and expressed as the mean \pm S.E. ($n = 7-8$). Significant differences between injections at each time point (0, 30, 60 min, etc.) were analyzed using one-way analysis of variance following post hoc Tukey Honestly Significant Difference and least significant difference tests. Student's *t* test (two-tailed) was used to determine the effects of MDMA on 5-HT and

5-HIAA concentrations. Data are reported as significant at $p < 0.05$ and were analyzed using SPSS version 11 (SPSS Inc., Chicago, IL).

Results

MDMA-Mediated 5-HT Release Is Progressively Attenuated upon Sequential Dosing. Consistent with published literature, MDMA (20 mg/kg s.c.) causes an acute release of 5-HT in the striatum of freely moving rats (Fig. 2). 5-HT release was maximal (0.68 pmol/ μ L) during the first sampling period (0–30 min) and subsequently declined to control levels between 210 and 240 min after drug administration (Fig. 2). Subsequent injection of the same dose of MDMA caused a similar increase, peak concentration, and decline in striatal 5-HT concentrations (Fig. 2). However, peak 5-HT concentrations were attenuated (relative to the first dose) after the third and fourth doses of MDMA, and baseline levels of 5-HT were reached between 180 and 210 min after drug administration (Fig. 2). Estimation of the $AUC_{0-300 \text{ min}}$ revealed that extracellular concentrations of 5-HT decreased $\sim 42\%$ between the first and fourth doses, and statistically significant declines in 5-HT release were evident following the third and fourth doses of MDMA, the second dose causing a modest but statistically insignificant decline compared with that produced by the initial dose of MDMA (Fig. 2). To confirm that the dosing protocol used in these studies produced serotonergic neurotoxicity, 5-HT and 5-HIAA concentrations were determined in the striatum and cortex 7 days after the last dose of MDMA. Consistent with previous reports from our laboratory (and others), MDMA (4×20 mg/kg s.c.) caused a 66 and 45% decrease in striatal and cortical 5-HT concentrations (Fig. 3A), respectively, and a 54 and 39% decrease in striatal and cortical 5-HIAA concentrations (Fig. 3B).

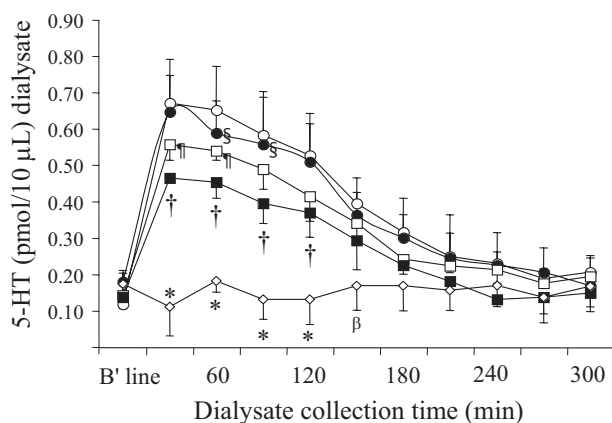


Fig. 2. MDMA-induced 5-HT release into striatal dialysate is attenuated following multiple s.c. injections. Animals received four injections of MDMA (20 mg/kg s.c.) at 12-h intervals. Two 30-min baseline samples were collected before each treatment of MDMA (20 mg/kg s.c.). Dialysate samples were analyzed by HPLC-CEAS as described under *Materials and Methods*. The data represent striatal concentrations of 5-HT following saline administration (\diamond) or following the first (\circ), second (\bullet), third (\square), and fourth (\blacksquare) injections of MDMA. Results are expressed as mean \pm S.E. ($n = 7-8$). Control values significantly different ($p < 0.05$) from values following: *, all four injections; β , the first three injections. Values for the fourth injection significantly different ($p < 0.05$) from values following the first (\dagger), second (\S), or third (\P) injections.

Thioether Metabolites of *N*-Me- α -MeDA Accumulate in the Striatum following Sequential Dosing with MDMA. Administration of MDMA (20 mg/kg s.c.) at 12-h intervals for a total of four injections led to a significant accumulation of the *N*-Me- α -MeDA thioether metabolites of MDMA in striatal dialysate (Figs. 4 and 5). Thus, following the first dose of MDMA, 5-(glutathion-*S*-yl)-*N*-Me- α -MeDA (Fig. 4A) and 2,5-bis-(glutathion-*S*-yl)-*N*-Me- α -MeDA (Fig. 4B) were detected in the first sampling of striatal dialysate (0–30 min), and peak concentrations of both GSH conjugates were reached during the second sampling period, 30 to 60 min after drug injection. Although the C_{\max} values for 5-(glutathion-*S*-yl)-*N*-Me- α -MeDA were similar after each dose (Table 1) and were followed by a rapid decline, the $AUC_{0-300 \text{ min}}$ increased $\sim 33\%$ between the first and fourth injections. C_{\max} values for 2,5-bis-(glutathion-*S*-yl)-*N*-Me- α -MeDA varied from 29 to 40 pmol/10 μ L after each dose, but the $AUC_{0-300 \text{ min}}$ essentially doubled between the first and fourth injections (Table 1). The rate of decline in striatal concentrations of 5-(glutathion-*S*-yl)-*N*-Me- α -MeDA ($t_{1/2}$, 85–113 min) and 2,5-bis-(glutathion-*S*-yl)-*N*-Me- α -MeDA ($t_{1/2}$, 82–104 min) were similar after each dose of MDMA (Fig. 4, A and B; Table 1).

Maximal concentrations of 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA and 2,5-bis-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA occurred between 2.3 and 3.6 h after drug injections (Fig. 5, A and B; Table 1). Because both mercapturic acid metabolites could still be detected in striatal dialysate 12 h after the preceding injections, $\frac{1}{2} C_{\max}$ values for 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA and 2,5-bis-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA were reached progressively earlier following subsequent injections. Thus, the time to $\frac{1}{2} C_{\max}$ for 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA decreased by 45% between the first (124 min) and fourth (68 min) injections and for 2,5-bis-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA decreased by 80% between the first (85 min) and fourth (17.5 min) injections. Although the C_{\max} values for 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA (36–42 pmol/10 μ L) were essentially the same following each injection, the C_{\max} values for 2,5-bis-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA increased $\sim 50\%$, from 36 to 53 pmol/10 μ L between the first and fourth injections. Accumulation of the mercapturic acid metabolites in the striatum was reflected by increases in the $AUC_{0-300 \text{ min}}$ for both 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA (35%) and 2,5-bis-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA (85%) between the first and fourth doses (Table 1), probably because processes for their elimination become saturated. Indeed, the elimination half-life of 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA (2.65 to 4.05 h) and 2,5-bis-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA (4.88 to 6.25h) increased by 53 and 28%, respectively, between the first and third doses (Fig. 5; Table 1). In fact, striatal concentrations of 2,5-bis-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA immediately before the third and fourth injections of MDMA (12 h after the preceding injection) are essentially the same as those measured 90 min after the first injection. The combined concentration of the four thioether metabolites in striatal dialysate (Fig. 6) reached a maximum of ~ 130 pmol/10 μ L (13 μ M) after the third and fourth injections, and the $AUC_{0-300 \text{ min}}$ increased 63% between the first and fourth doses. It is noteworthy that total concentrations remained relatively unchanged for ~ 4 h after exposure to MDMA (Fig. 6), indicating that serotonergic neurons are exposed to these

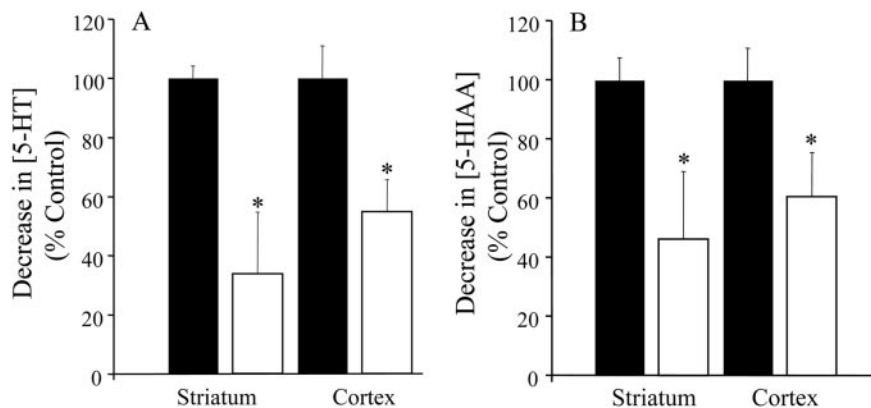


Fig. 3. Effect of MDMA on 5-HT (A) and 5-HIAA (B) concentrations in striatum and cortex. 5-HT (A) and 5-HIAA (B) concentrations were determined 7 days following the last of four injections of MDMA (20 mg/kg s.c.) by HPLC-CEAS as described under *Materials and Methods*. Absolute values for 5-HT and 5-HIAA concentration in control animals were as follows: striatum = 2.48 ± 0.32 and 2.19 ± 0.25 pmol/mg tissue, respectively; and cortex = 1.12 ± 0.22 and 1.09 ± 0.35 pmol/mg tissue, respectively. Data represent vehicle treated controls (black bars) or MDMA-treated animals (open bars). The percentage decreases in 5-HT and 5-HIAA concentrations are expressed as the mean \pm S.E. ($n = 7-8$ in each group). *, values from MDMA-treated animals are significantly different ($p < 0.05$) from vehicle-treated control values.

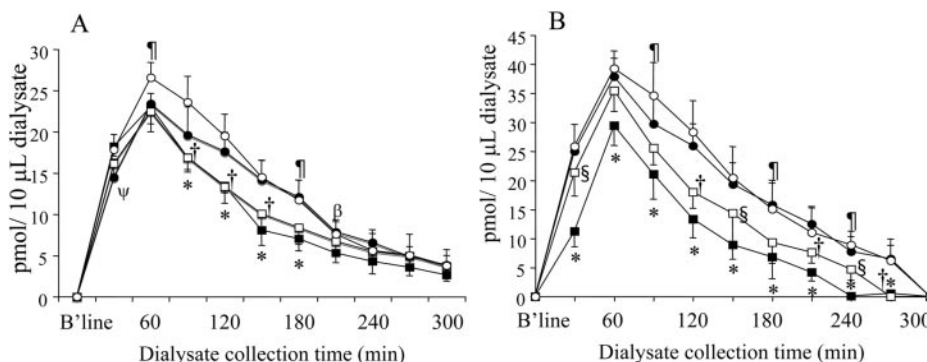


Fig. 4. Striatal concentrations of 5-(glutathion-S-yl)-N-Me- α -MeDA (A) and 2,5-bis-(glutathion-S-yl)-N-Me- α -MeDA (B) increase following multiple injections of MDMA. Animals were injected four times with MDMA (20 mg/kg s.c.) at 12-h intervals, with two-30 min "baseline" samples collected immediately before each treatment. Dialysate samples were then collected every 30 min for 5 h following each injection, and concentrations of 5-(glutathion-S-yl)-N-Me- α -MeDA (A) and 2,5-bis-(glutathion-S-yl)-N-Me- α -MeDA (B) were determined by HPLC-CEAS as described under *Materials and Methods*. Results are expressed as means \pm S.E. ($n = 7-8$) and represent data from the first (■), second (□), third (●), and fourth (○) s.c. injections of MDMA. Values for the first injection were significantly different ($p < 0.05$) from the injections noted as follows: *, both the third and fourth injections; †, both the third and fourth injections; ‡, both the third and fourth injections; §, second injection; ¶, third injection; ⋈, fourth injection only. Values for second injection were also significantly different ($p < 0.05$) from the following: †, both the third and fourth injections; and ‡, fourth injection only.

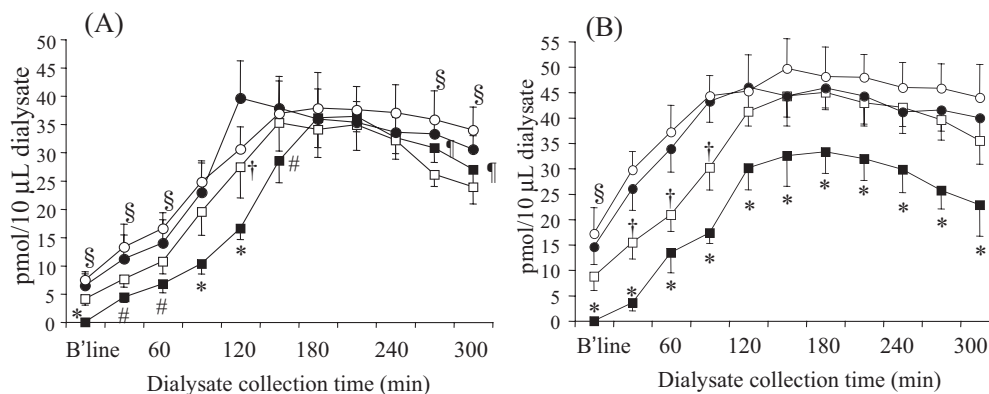


Fig. 5. 5-(N-acetylcystein-S-yl)-N-Me- α -MeDA (A) and 2,5-bis-(N-acetylcystein-S-yl)-N-Me- α -MeDA (B) accumulate and persist in the striatum of rats following multiple injections of MDMA. Animals were injected four times with MDMA (20 mg/kg s.c.) at 12-h intervals, with two-30 min baseline samples collected immediately before each treatment. Dialysate samples were then collected every 30 min for 5 h following each injection, and concentrations of 5-(N-acetylcystein-S-yl)-N-Me- α -MeDA and 2,5-bis-(N-acetylcystein-S-yl)-N-Me- α -MeDA were determined by HPLC-CEAS as described under *Materials and Methods*. Results are expressed as means \pm S.E. ($n = 7-8$) and represent data from the first (■), second (□), third (●), and fourth (○) s.c. injections of MDMA. Values for the first injection were significantly different ($p < 0.05$) from the injections noted as follows: *, second, third, and fourth injections; †, third and fourth injections; ‡, fourth injection only. Values for second injection were also significantly different ($p < 0.05$) from the following: †, both the third and fourth injections; and ‡, fourth injection only.

neurotoxic metabolites for a prolonged period of time. A significant ($p < 0.05$) positive correlation was observed among striatal 5-(glutathion-S-yl)-N-Me- α -MeDA, 5-(N-acetylcystein-S-yl)-N-Me- α -MeDA, and total thioether concentrations (fourth dose AUC_{0-300 min}) and MDMA-mediated neurotoxicity, assessed by decreases in 5-HT concentrations 7 days after the last dose of MDMA.

Discussion

Neurotoxic thioether metabolites of MDMA accumulate in the striatum of rats following multiple dosing (Figs. 4 and 5). These data have important implications because the desired pharmacological effects of MDMA last between 3 and 6 h after dosing, and users of the drug usually ingest multiple

TABLE 1

Pharmacokinetics of the thioether metabolites of *N*-Me- α -MeDA in rat striatum following multiple injections of MDMA (20 mg/kg s.c.)

Thioether Metabolites of <i>N</i> -Me- α -MeDA	C_{max}	$t_{1/2}$	T_{max}	AUC _{0-300 min}
	pmol/10 μ l	min		pmol/ μ l \times min
5-(GSyl)-<i>N</i>-Me-α-MeDA				
Dose 1	23 \pm 2	85 \pm 10	60 \pm 0	303 \pm 10
Dose 2	23 \pm 2	110 \pm 25	60 \pm 0	319 \pm 18
Dose 3	24 \pm 2	113 \pm 21	63 \pm 10	369 \pm 19*†
Dose 4	27 \pm 2	97 \pm 28	60 \pm 0	402 \pm 32*†
2,5-bis(GSyl)-<i>N</i>-Me-α-MeDA				
Dose 1	29 \pm 3	90 \pm 30	60 \pm 0	287 \pm 28
Dose 2	35 \pm 4	82 \pm 19	60 \pm 0	402 \pm 28*
Dose 3	38 \pm 3	104 \pm 21	60 \pm 0	532 \pm 63*†
Dose 4	40 \pm 3	95 \pm 18	63 \pm 10	560 \pm 74*†
5-(NACSyl)-<i>N</i>-Me-α-MeDA				
Dose 1	38 \pm 4	159 \pm 20	200 \pm 33	648 \pm 24
Dose 2	36 \pm 4	212 \pm 19	167 \pm 37	726 \pm 75
Dose 3	41 \pm 6	243 \pm 23	137 \pm 30	846 \pm 94*†
Dose 4	42 \pm 5		217 \pm 42	873 \pm 65*†
2,5-bis(NACSyl)-<i>N</i>-Me-α-MeDA				
Dose 1	36 \pm 4	293 \pm 34	170 \pm 30	687 \pm 47
Dose 2	49 \pm 2	325 \pm 27	180 \pm 30	1032 \pm 41*
Dose 3	49 \pm 1	375 \pm 34	143 \pm 39	1179 \pm 31*†
Dose 4	53 \pm 4		170 \pm 40	1273 \pm 73*†‡

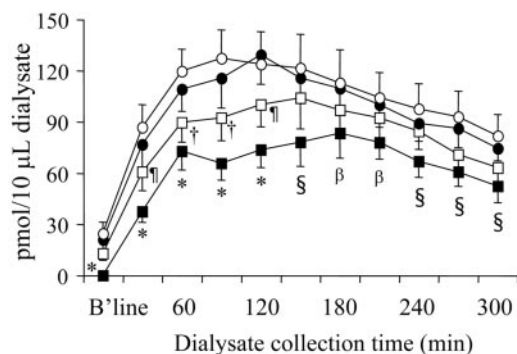
* Significantly different ($p < 0.05$) from AUC following the first injection of MDMA.† Significantly different ($p < 0.05$) from AUC following the second injection of MDMA.‡ Significantly different ($p < 0.05$) from AUC following the third injection of MDMA.

Fig. 6. Thioether metabolites of *N*-Me- α -MeDA accumulate and persist in the striatum of rats following multiple injections of MDMA. Animals were injected four times with MDMA (20 mg/kg s.c.) at 12-h intervals, with two-30 min baseline samples collected immediately before each treatment. Dialysate samples were then collected every 30 min for 5 h following each injection, and concentrations of the various thioether metabolites were determined by HPLC-CEAS as described under *Materials and Methods*. Results are expressed as means \pm S.E. ($n = 7-8$) of the combined metabolites and represent data from the first (■), second (□), third (●), and fourth (○) s.c. injections of MDMA. Values for the first injection were significantly different ($p < 0.05$) from the second, third, and fourth injections (*), the third and fourth injections only (§), and the fourth injection only (¶). Values for the second injection were significantly different ($p < 0.05$) from both the third and fourth injections (†) and from the fourth injection only (‡).

doses during the course of a rave or at similar recreational gatherings. Party raves are frequented by those who combine the ingestion of MDMA with overnight or weekend-long dancing and typify the manner in which this drug is used for "recreational" purposes (see *Materials and Methods*). Initial studies demonstrating that *N*-Me- α -MeDA thioethers are present in striatal dialysate of rats receiving s.c. MDMA (Jones et al., 2005) also revealed that the mercapturic acid metabolites of *N*-Me- α -MeDA were cleared relatively slowly from the brain. These data, together with earlier findings from our laboratory that investigated the disposition of i.c.v. administered 5-(glutathion-*S*-yl)- α -MeDA (Miller et al., 1996), a metabolite of MDA, raised the possibility that mul-

iple dosing with MDMA may lead to the accumulation of these metabolites to concentrations sufficiently high as to produce neurotoxicity. The present studies substantiated this possibility.

Hepatic formation of GSH conjugates of α -MeDA and *N*-Me- α -MeDA, followed by uptake into and metabolism by the brain, provides a potential mechanism to explain the role of metabolism in MDA- and MDMA-mediated neurotoxicity (Monks et al., 2004). Systemic formation of *N*-Me- α -MeDA thioether conjugates requires that a mechanism for conjugate transport across the blood-brain barrier be present. There appears to be a transporter capable of transferring GSH and GSH conjugates from the circulation into the brain (Kannan et al., 1998). Indeed, the uptake of 5-(glutathion-*S*-yl)- α -Me[3 H]DA into brain decreases in the presence of GSH, suggesting that the two compounds share the same transport mechanism (Miller et al., 1995). In contrast, inhibition of γ -glutamyltranspeptidase (γ -GT) with acivicin within brain microvessel endothelial cells at the blood brain barrier potentiates the uptake of 5-(glutathion-*S*-yl)- α -Me[3 H]DA into the brain (Miller et al., 1995) and exacerbates MDMA/MDA (10 mg/kg)-mediated depletions in 5-HT and 5-HIAA concentrations (Bai et al., 2001). These data provide evidence that the serotonergic neurotoxicity observed following peripheral administration of MDA or MDMA requires the synthesis of metabolites that serve as substrates for γ -GT. This effect is therefore probably due to decreases in the metabolic clearance of GSH conjugates of α -MeDA and *N*-Me- α -MeDA within the brain capillary lumen, resulting in increases in intact 5-(glutathion-*S*-yl)- α -MeDA concentrations available for uptake across microvessel endothelial cells via the GSH transporter. These findings have important implications because structurally related polyphenolic-GSH conjugates decrease the activity of renal γ -GT (Hill et al., 1991). Prolonged exposure of the blood brain-barrier to α -MeDA and *N*-Me- α -MeDA-thioethers might therefore be expected to decrease γ -GT activity, with a subsequent increase in the uptake of the neurotoxic conjugates into brain. Consistent with this

view, quinone-thioethers have been shown to inhibit a variety of enzymes that utilize GSH as either a substrate or cosubstrate (Monks and Lau, 1998).

Residency of *N*-Me- α -MeDA-thioethers within the brain will be dependent upon a variety of competing pathways, of which metabolite efflux will be a major determinant. There are a host of proteins that participate in the transport of drugs and their metabolites across biological membranes (Deeley et al., 2006). Export from the brain involves two major pathways, transport across endothelial cells into blood and transport into cerebrospinal fluid mediated by the epithelial cells that comprise the choroid plexus. Remarkably little is known about the export of GSH conjugates from the brain, and essentially nothing has been reported with respect to the export of mercapturic acids, although information is available on organic anion transport in general. The cellular export of GSH and GSH conjugates is mediated by a subset of proteins belonging to the ATP-binding cassette superfamily of transport proteins (Cole and Deeley, 2006). Several members of the ATP-binding cassette subfamily C [also known as the multidrug resistance-associated proteins (MRPs)] mediate the cellular efflux of GSH and GSH conjugates. Of the various MRPs, knowledge on substrates for MRP1 is the most extensive, and the protein is expressed in brain endothelial cells. In addition to playing an important role in restricting the access of drugs and other xenobiotics to the brain (Deeley et al., 2006) and in limiting the efficacy of drug treatment to drug-resistant tumor tissue (Rappa et al., 1999), MRP is also a major mediator of the efflux of GSH-xenobiotic conjugates (Cole and Deeley, 2006) and as such could play a major role in limiting the residency time of such conjugates within the brain. Indeed, our studies on the disposition of i.c.v. administered 5-(glutathion-*S*-yl)- α -MeDA revealed that concentrations of this metabolite decrease rapidly (Miller et al., 1995). However, decreases in the concentration of 5-(glutathion-*S*-yl)- α -MeDA were accompanied by increases in the concentration of the corresponding cysteine and *N*-acetylcysteine conjugates, suggesting that both export from and metabolism within brain parenchyma contribute to the elimination of 5-(glutathion-*S*-yl)- α -MeDA from the brain. In contrast to the GSH conjugates, the mercapturic acid metabolites of *N*-Me- α -MeDA (Fig. 5) and α -MeDA (Miller et al., 1995) are only slowly eliminated from the brain. Because these metabolites are the most potent serotonergic neurotoxins of the various *N*-Me- α -MeDA thioethers, their persistence in brain following systemic administration of MDMA will have functional consequences. Organic anions are substrates for three major classes of transport proteins, the organic anion transport (OAT) family, the organic anion-transporting polypeptide family, and the MRP family (Hagenbuch and Meier, 2003; Sekine et al., 2006). Although much is known about the renal physiology of the OATs and organic anion-transporting peptides, comparatively little is known about the localization and function of these proteins in brain. OAT1 and OAT3 have been identified in human and rat brain, and cysteine conjugates are a reported substrate for OAT1 (Sekine et al., 2006). The *N*-acetylcysteine conjugate of MeHg is a poor substrate for OAT3 but is effectively transported by OAT1 (Koh et al., 2002). The role of OAT1 in the transport and neurotoxicity of 5-(*N*-acetylcystein-*S*-yl)-*N*-Me- α -MeDA is under investigation.

5-(*N*-Acetylcystein-*S*-yl)- α -MeDA, 5-(glutathion-*S*-yl)- α -

MeDA, and 2,5-bis-(glutathion-*S*-yl)- α -MeDA produce neurobehavioral changes in rats characteristic of peripheral administration of MDMA/MDA as well as acute increases in brain 5-HT and DA concentrations (Miller et al., 1996). The finding that α -MeDA itself did not produce the same behavioral profile as 5-(glutathion-*S*-yl)- α -MeDA (Miller et al., 1996) or 2,5-bis-(glutathion-*S*-yl)- α -MeDA (Miller et al., 1997) suggest that the behavioral changes are not simply a consequence of the catecholamine function. In this respect, GSH and related enzymes participate in the protection of neurons from a variety of stresses, and changes in GSH metabolism have been associated with neurodegenerative processes of the brain, such as ischemia, Parkinson's disease, and Alzheimer's disease. However, additional roles for GSH in brain function are being reported (Shaw, 1998). The unique structure of catecholamine-GSH conjugates such as 5-(glutathion-*S*-yl)-*N*-Me- α -MeDA and α -MeDA permits toxicological and pharmacological activity as a consequence of either the catecholamine or GSH moiety. Neuropharmacological changes may result from either the catecholamine or GSH moiety, whereas toxicological sequelae may result from the electrophilic and redox properties of the quinone function. Indeed, 5-(glutathion-*S*-yl)- α -MeDA and 5-(*N*-acetylcystein-*S*-yl)- α -MeDA inhibit human 5-HT transporter function and simultaneously stimulate dopamine uptake into human 5-HT transporter-expressing SK-N-MC cells (Jones et al., 2004).

In summary, thioether metabolites accumulate in rat brain following multidose administration of MDMA, primarily as a consequence of the persistence in brain of the mercapturic acid metabolites of *N*-Me- α -MeDA. The basis for the slow rate of elimination of these organic anions from rat brain is unclear. However, the ability of these metabolites to generate reactive oxygen species and to arylate proteins, in combination with their ability to modulate the activity of proteins involved in the regulation of neurotransmitter transport (Jones et al., 2004), suggest they play an important role in the development of MDMA-mediated serotonergic neurotoxicity.

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