# Glutathione and *N*-Acetylcysteine Conjugates of α-Methyldopamine Produce Serotonergic Neurotoxicity: Possible Role in Methylenedioxyamphetamine-Mediated Neurotoxicity

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Direct injection of either 3,4-(±)-methylenedioxymethamphetamine (MDMA) or 3,4-(±)-methylenedioxyamphetamine (MDA) into the brain fails to reproduce the serotonergic neurotoxicity seen following peripheral administration. The serotonergic neurotoxicity of MDA and MDMA therefore appears to be dependent upon the generation of a neurotoxic metabolite, or metabolites, the identity of which remains unclear. α-Methyldopamine (α-MeDA) is a major metabolite of both MDA and MDMA. We have shown that intracerebroventricular (icv) injection of 2,5-bis(glutathion-*S*-yl)-α-methyldopamine [2,5-bis(glutathion-*S*-yl)-α-MeDA] causes decreases in serotonin concentrations in the striatum, cortex, and hippocampus, and neurobehavioral effects similar to those seen following MDA and MDMA administration. In contrast, although 5-(glutathion-*S*-yl)-α-methyldopamine [5-(glutathion-*S*-yl)-α-MeDA] and 5-(*N*-acetylcystein-*S*-yl)-α-methyldopamine [5-(*N*-acetylcystein-*S*-yl)-α-MeDA] produce neurobehavioral changes similar to those seen with MDA and MDMA, and acute changes in brain 5-HT and dopamine concentrations, neither conjugate caused long-term decreases in 5-HT concentrations. We now report that direct intrastriatal or intracortical administration of 5-(glutathion-*S*-yl)-α-MeDA (4 × 200 or 4 × 400 nmol), 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (4 × 7 or 4 × 20 nmol), and 2,5-bis(glutathion-*S*-yl)-α-MeDA (4 × 150 or 4 × 300 nmol) causes significant decreases in striatal and cortical 5-HT concentrations (7 days following the last injection). Interestingly, intrastriatal injection of 5-(glutathion-*S*-yl)-α-MeDA or 2,5-bis(glutathion-*S*-yl)-α-MeDA, but not 5-(*N*-acetylcystein-*S*-yl)-α-methyldopamine, also caused decreases in 5-HT concentrations in the ipsilateral cortex. The same pattern of changes was seen when the conjugates were injected into the cortex. The effects of the thioether conjugates of α-MeDA were confined to 5-HT nerve terminal fields, since no significant changes in monoamine neurotransmitter levels were detected in brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla). In addition, the effects of the conjugates were selective with respect to the serotonergic system, as no significant changes were seen in dopamine or norepinephrine concentrations. The results indicate that thioether conjugates of α-MeDA are selective serotonergic neurotoxicants. Nonetheless, a role for these conjugates in the toxicity observed following systemic administration of MDA and MDMA remains to be demonstrated, and requires further experimentation.

## Introduction

3,4-(±)-Methylenedioxyamphetamine (MDA)[1] and 3,4-(±)-methylenedioxymethamphetamine (MDMA, "Ecstasy") are ring-substituted amphetamine derivatives that have stimulant and hallucinogenic properties (*1*, *2*). MDA and MDMA are popular recreational drugs, and their abuse is increasing in both the United States (*3*) and Europe (*4*). The predominant adverse consequences of MDMA and MDA abuse in humans include convulsions, hyperthermia, rhabomyolysis, and acute liver and renal failure (*5*). In experimental animals, including primates, toxicity is also manifest as a selective serotonergic neurotoxicity, featuring an acute release of 5-hydroxytryptamine (5-HT) followed by prolonged depletion of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels (*6*, *7*), inhibition of tryptophan hydroxylase (TPH) (*7*, *8*), and structural damage to serotonergic nerve terminals (*9*−*13*).

The neurotoxic effects of MDA and MDMA are dependent on the route and frequency of drug administration (*14*). Direct injection of MDA or MDMA into the brain does not reproduce the acute or long-term effects observed after peripheral administration, suggesting an important role for systemic metabolism in the development of

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[1] Abbreviations: ACSF, artificial cerebrospinal fluid; CEAS, coulometric electrode array system; P450, cytochrome P450; DA, dopamine; DOPAC, (3,4-dihydroxyphenyl)acetic acid; EDTA, disodium ethylenediaminetetraacetate; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine (serotonin); icv, intracerebroventricular; MDA, 3,4-(±)-methylenedioxyamphetamine; MDMA, 3,4-(±)-methylenedioxymethamphetamine; α-MeDA, α-methyldopamine; 5-(glutathion-*S*-yl)-α-MeDA, 5-(glutathion-*S*-yl)-α-methyldopamine; 5-(*N*-acetylcystein-*S*-yl)-α-MeDA, 5-(*N*-acetylcystein-*S*-yl)-α-methyldopamine; 2,5-bis(glutathion-*S*-yl)-α-MeDA, 2,5-bis(glutathion-*S*-yl)-α-methyldopamine; NE, norepinepherine; ROS, reactive oxygen species; TPH, tryptophan hydroxylase.

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toxicity (*15*−*17*). In support of this view, pretreatment of rats with SKF-525A, an inhibitor of cytochrome P450 (P450), attenuates MDMA-mediated depletions in 5-HT levels, whereas pretreatment with phenobarbital enhances 5-HT depletion (*18*). Moreover, the inability of MDMA to inhibit TPH activity in vitro supports a requirement for metabolic activation (*16*). However, several major metabolites of MDA and MDMA either fail to reproduce the serotonergic neurotoxicity or fail to exhibit specificity for the serotonergic system (*19*, *20*).

α-Methyldopamine (α-MeDA) is a metabolite of MDA and MDMA (*21*, *22*). P450 2D, P450 2B, and P450 3A1 all catalyze the demethylenation of MDA to form α-MeDA (*23*). However, intracerebroventricular (icv) administration of α-MeDA to rats produces neither the acute "serotonin syndrome" (*24*) nor long-term depletions in 5-HT levels (*19*). α-MeDA undergoes oxidation to the corresponding *o*-quinone, which is efficiently scavenged by glutathione (GSH) (*25*, *26*) to form 5-(glutathion-*S*-yl)-α-methyldopamine [5-(glutathion-*S*-yl)-α-MeDA]. Icv administration of 5-(glutathion-*S*-yl)-α-MeDA results in the rapid formation of 5-(cystein-*S*-yl)-α-methyldopamine [5-(cystein-*S*-yl)-α-MeDA] and 5-(*N*-acetylcystein-*S*-yl)-α-methyldopamine [5-(*N*-acetylcystein-*S*-yl)-α-MeDA] (*27*). 5-(Glutathion-*S*-yl)-α-MeDA is also readily oxidized to the *o*-quinone GSH conjugate, and undergoes addition of a second molecule of GSH to form 2,5-bis(glutathion-*S*-yl)-α-MeDA (*24*). Electrochemical studies indicate that the thioether conjugates of α-MeDA exhibit lower half-wave oxidation potentials ($E_{1/2}$) than α-MeDA (*27*) and thus are potentially more reactive.

The ability of polyphenolic thioether conjugates to redox cycle and produce reactive oxygen species (ROS) provides a rationale for the potential role of these metabolites in MDA and MDMA neurotoxicity. Consistent with this hypothesis, icv injection of 2,5-bis(glutathion-*S*-yl)-α-MeDA causes selective decreases in 5-HT concentrations in the striatum, cortex, and hippocampus, and neurobehavioral effects identical to those seen following MDA and MDMA administration (*24*). In contrast, although 5-(glutathion-*S*-yl)-α-MeDA and 5-(*N*-acetylcystein-*S*-yl)-α-MeDA produce neurobehavioral changes similar to those seen with MDA and MDMA, and acute changes in brain 5-HT and dopamine concentrations, neither conjugate causes long-term decreases in 5-HT concentrations (*24*, *28*). Because of the inherent reactivity of polyphenolic thioethers, icv injection may result in concentrations in target areas that are insufficient to produce toxicity. We therefore determined the effects of 2,5-bis(glutathion-*S*-yl)-α-MeDA, 5-(glutathion-*S*-yl)-α-MeDA, and 5-(*N*-acetylcystein-*S*-yl)-α-MeDA on monoaminergic neurotransmitter concentrations following their direct injection into the striatum, cortex, and hippocampus.

## Materials and Methods

**Chemicals.** GSH, mushroom tyrosinase (5600 units/mg), 5-HT, 5-HIAA, dopamine (DA), and norepinephrine (NE) were obtained from Sigma Chemical Co. (St. Louis, MO). α-MeDA was a generous gift from A. Y. H. Lu (Merck Research Laboratories, Rahway, NJ). (±)-MDA was kindly provided by the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD). 2,5-Bis(glutathion-*S*-yl)-α-MeDA, 5-(glutathion-*S*-yl)-α-MeDA, and 5-(*N*-acetylcystein-*S*-yl)-α-MeDA were synthesized as previously described (*24*, *28*, *29*).

**Animals.** Male Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX; 200−225 g) were used in all the experiments. The rats were housed in the Animal Resource Center at the University of Texas at Austin, and maintained on a 12 h light/dark cycle and at a constant room temperature (72 °F). Food and water were provided ad libitum.

**Intrastriatal, Intracortical, and Intrahippocampal Cannula Implantation.** Surgeries were performed to implant a guide cannula into rat left striatum, left cortex, or left hippocampus. Rats were anesthetized with 3.5 mL of Equithesin/kg containing sodium phenobarbital (9.4 mg/mL) and chloral hydrate (37.5 mg/mL), and heads were shaved and placed in a stereotaxic apparatus. A midsaggital incision was made to expose the skull. The skull was leveled, and a small burr hole was made with a hand drill. The stereotaxic coordinates for implantation of the cannulae were as follows: (−) 0.8 mm from bregma, (+) 3.0 mm lateral to the midline for striatum; (−) 4 mm from bregma, (+) 3.0 mm lateral to the midline for cortex; and (−) 4.5 mm from bregma, (+) 3.0 mm lateral to the midline for hippocampus. A 26 g guide cannula (Plastic One, Roanoke, VA) was lowered either 4.5 (striatum), 1.2 (cortex), or 2.9 mm (hippocampus) below the surface of the skull to guide an injection probe, which was cut 1.5 mm below the bottom of the guide cannula. Three additional burr holes were made to attach jeweler's screws. Cranioplastic (Plastic One) was spread over the area of the guide cannula and anchor screws and allowed to dry. A dummy cannula was inserted into the guide cannula. The incision was closed by sutures and Neosporin applied to the incision. Rats were allowed to recover for 1 week before drug administration.

**Drug Administration.** 5-(Glutathion-*S*-yl)-α-MeDA (200 and 400 nmol), 2,5-bis(glutathion-*S*-yl)-α-MeDA (150 and 300 nmol), and 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (7 and 20 nmol) were administered four times at 12 h intervals. This multiple-dosing strategy was employed to mimic the majority of protocols used to investigate MDA- and MDMA-mediated serotonergic toxicity. All conjugates were dissolved in 1 μL of artificial cerebrospinal fluid (ACSF; 147 mM NaCl, 4 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgSO_4$). Each α-MeDA thioether was infused into the left striatum, cortex, or hippocampus of the awake freely moving animal at a rate of 0.2 μL/min using a Hamilton syringe connected to the injection probe. The injection probe was left in the guide cannula for 1−2 min after the injection. A dummy cannula was inserted into the guide cannula to close the injection site each time after the injection. Control animals were administered an equal volume of ACSF. MDA was used as a positive control and was administered to rats peripherally (93 μmol/kg, sc). Seven days after the last injection, rats were euthanized by decapitation and their brains quickly removed and placed onto an ice-cold plate. Brain regions corresponding to striatum, cortex, hippocampus, midbrain/diencephalon/telencephalon, and pons/medulla were dissected free and frozen by liquid nitrogen in preweighed microcentrifuge tubes. Rat brains were dissected as described above to obtain brain regions enriched in 5-HT nerve terminals and major targets of MDA neurotoxicity (striatum, cortex, and hippocampus) and brain regions containing 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla). Brain tissue was stored at −80 °C for no longer than 1 week prior to analysis. For neurotransmitter analyses, tissue was weighed and sonicated with a sonic dismembranator (Fisher Sci.) in ice-cold 0.1 N $HClO_4$ containing 134 μM EDTA and 263 μM $Na_2S_2O_5$ for 30 s. The sonicated tissues were centrifuged at 13500*g* (4 °C) for 10 min. Supernatants were centrifuged again under the same condition and aliquots (20 μL) of this supernatant used for HPLC analysis.

**HPLC−CEAS Monoamine Neurotransmitter Analyses.** 5-HT, 5-HIAA, DA, and NE levels were quantified by HPLC equipped with an eight-channel coulometric electrode array system (HPLC−CEAS; ESA Inc., Chelmsford, MA). The potentials applied to the electrodes increased in 50 mV increments, starting from 0 mV at the first channel, and increasing to 350

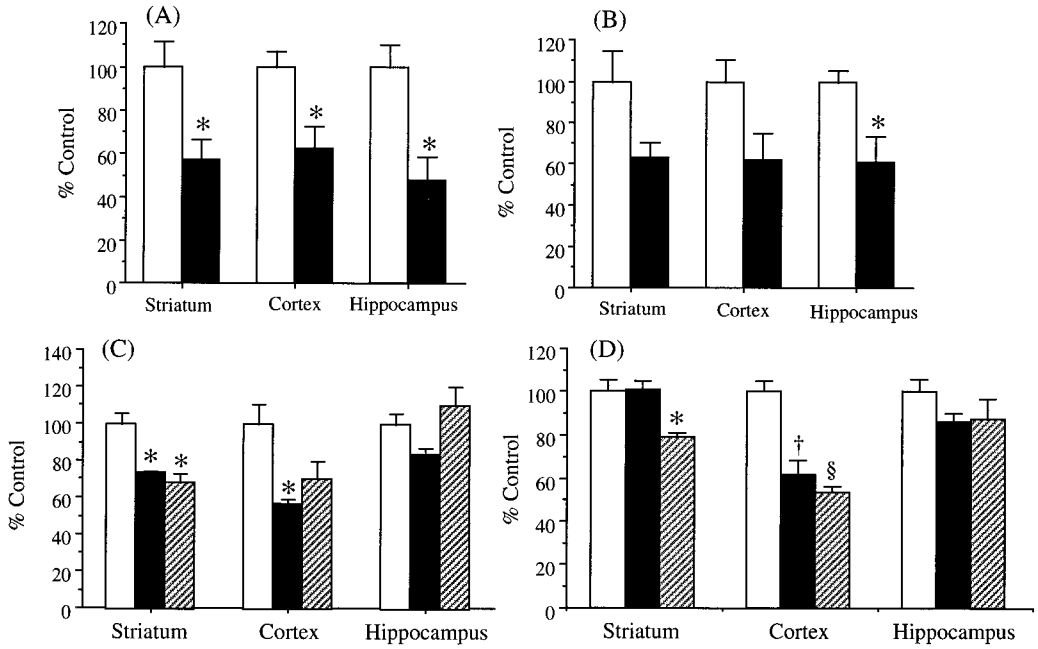


**Figure 1.** Effect of MDA (93 $\mu$mol/mg, sc) and 5-(glutathion-*S*-yl)-α-MeDA (200 and 400 nmol) on serotonin and 5-HIAA concentrations in striatum, cortex, and hippocampus, 7 days after drug administration. Panels A and B show the effects of MDA on serotonin and 5-HIAA concentrations, respectively. The absolute values for 5-HT in the striatum, cortex, and hippocampus in control ACSF-treated animals (white bars) were 2.05 ± 0.24, 1.07 ± 0.08, and 2.16 ± 0.22 pmol/mg of tissue, respectively, and for 5-HIAA were 1.32 ± 0.19, 0.52 ± 0.06, and 1.23 ± 0.07 pmol/mg of tissue, respectively. Panel C shows the effects of 5-(glutathion-*S*-yl)-α-MeDA on serotonin concentrations following intrastriatal administration (200 nmol, black bars; 400 nmol, hatched bars) four times at 12 h intervals. The absolute values for 5-HT in the striatum, cortex, and hippocampus in control ACSF-treated animals (white bars) were 3.91 ± 0.23, 1.65 ± 0.18, and 2.53 ± 0.15 pmol/mg of tissue, respectively. Panel D shows the effects of 5-(glutathion-*S*-yl)-α-MeDA on serotonin concentrations following intracortical administration (200 nmol, black bars; 400 nmol, hatched bars) four times at 12 h intervals. The absolute values for 5-HT in the striatum, cortex, and hippocampus in control ACSF-treated animals (white bars) were 2.20 ± 0.12, 1.35 ± 0.07, and 1.05 ± 0.06 pmol/mg of tissue, respectively. Values are statistically significantly different from control values at the following levels: (*) $p < 0.05$, (†) $p < 0.01$, and (§) $p < 0.005$ ($n = 3$ for each group).

mV at the eighth channel. Sample aliquots were loaded onto an ESA HR-80 column (80 mm × 4.6 mm i.d., 3 mm particle size) and separated with a mobile phase consisting of 8 mM ammonium acetate, 4 mM citrate, 54 $\mu$M EDTA, 230 mM 1-octanesulfonic acid, and 5% methanol (pH 2.5). The flow rate was set at 1 mL/min. Quantitation of monoamine neurotransmitters and their metabolites was achieved by comparing the peak areas with standard curves generated from authentic standards.

**Data Analysis and Statistics.** Concentrations of monoamine neurotransmitters and their metabolites were expressed as means ± SE as picomoles per milligram of tissue (wet weight). The Student's *t* test was used to compare control to treated groups, and a $p$ of <0.05 was used to determine significant differences.

## Results

**Effects of 5-(Glutathion-*S*-yl)-α-MeDA on Monoamine Neurotransmitter Concentrations following Intrastriatal and Intracortical Administration.** As a positive control, and consistent with previously published data (*24*, *28*), striatal, cortical, and hippocampal 5-HT levels decreased by 43, 38, and 52%, respectively, 7 days after administration of MDA (93 $\mu$mol/kg, sc) (Figure 1A). The hippocampal 5-HIAA level also decreased significantly, to 61% of control values (Figure 1B). Concentrations of DA and NE were unaffected 7 days after administration (see the Supporting Information). Intrastriatal administration 5-(glutathion-*S*-yl)-α-MeDA (200 and 400 nmol) caused a decrease in 5-HT levels (Figure 1C) to 74 and 68% of controls, respectively ($p <$ 0.05). Concentrations of 5-HT in the striatum contralateral to the site of injection were unaltered. Interestingly, 5-HT concentrations in the ipsilateral cortex were also significantly ($p < 0.05$) decreased (Figure 1C). Hippocampal 5-HT concentrations were not affected. With the exception of increases in 5-HIAA concentrations in the striatum with 400 nmol of 5-(glutathion-*S*-yl)-α-MeDA, no significant changes in 5-HIAA concentrations were found. Brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla) were unaffected by the intrastriatal administration of 5-(glutathion-*S*-yl)-α-MeDA (see the Supporting Information). DA and NE concentrations did not change following intrastriatal administration of 5-(glutathion-*S*-yl)-α-MeDA (see the Supporting Information).

Intracortical administration of 5-(glutathion-*S*-yl)-α-MeDA (200 and 400 nmol) reduced 5-HT concentrations to 62 ($p < 0.01$) and 54% ($p < 0.005$) of control values (Figure 1D), respectively. Concentrations of 5-HT in the cortex contralateral to the site of injection were unaltered. Intracortical administration of 5-(glutathion-*S*-yl)-α-MeDA (400 nmol) also significantly ($p < 0.05$) decreased 5-HT concentrations in the striatum ipsilateral to the site of cortical injection (Figure 1D). Again, hippocampal 5-HT concentrations were not affected, and 5-HIAA concentrations in all brain regions that were examined were unchanged (see the Supporting Information). DA and NE concentrations did not change following intracortical administration of 5-(glutathion-*S*-yl)-α-MeDA (see the Supporting Information). Brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla) were unaffected by the intracortical administration of 5-(glutathion-*S*-yl)-α-MeDA (see the Supporting Information).

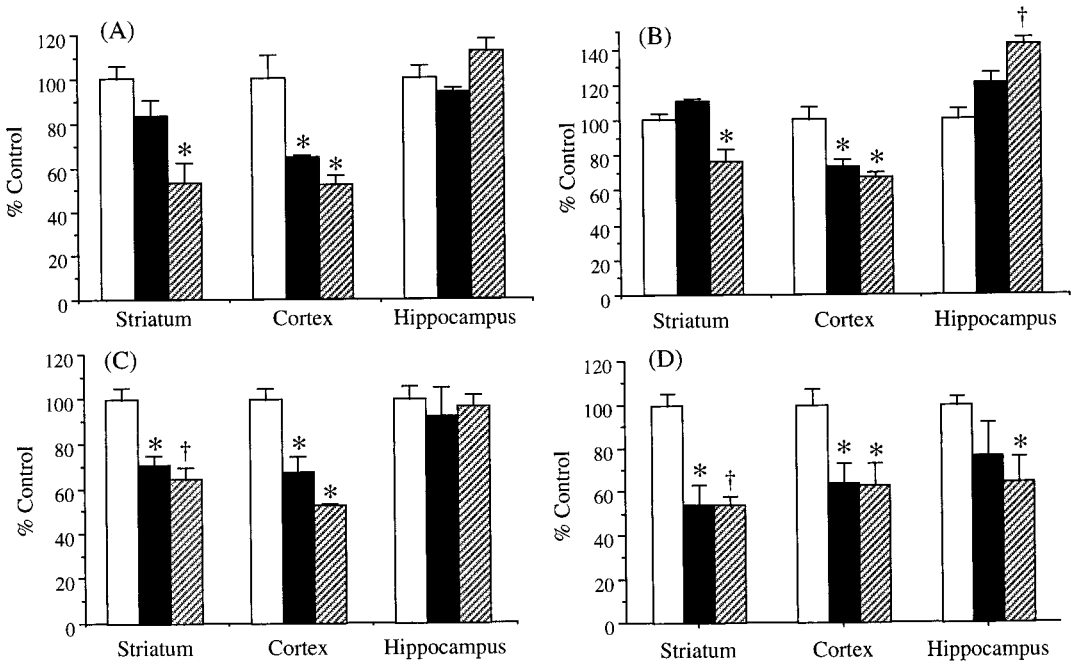


**Figure 2.** Effects of 2,5-bis(glutathion-*S*-yl)-α-MeDA (150 and 300 nmol) and 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (7 and 20 nmol) on 5-HT and 5-HIAA concentrations in striatum, cortex, and hippocampus, 7 days after drug administration. Panels A and B show the effects of 2,5-bis(glutathion-*S*-yl)-α-MeDA on 5-HT and 5-HIAA concentrations, respectively, following intrastriatal administration (150 nmol, black bars; 300 nmol, hatched bars) four times at 12 h intervals. The absolute values for 5-HT in the striatum, cortex, and hippocampus in control ACSF-treated animals (white bars) were 3.91 ± 0.23, 1.65 ± 0.18, and 2.53 ± 0.15 pmol/mg of tissue, respectively, and for 5-HIAA were 2.53 ± 0.09, 0.69 ± 0.05, and 1.38 ± 0.08 pmol/mg of tissue, respectively. Values are statistically significantly different from control values at the following levels: (*) $p < 0.05$ and (†) $p < 0.005$ ($n = 3$ for each group). Panel C shows the effects of 2,5-bis(glutathion-*S*-yl)-α-MeDA on 5-HT concentrations following intracortical administration (150 nmol, black bars; 300 nmol, hatched bars) four times at 12 h intervals. The absolute values for 5-HT in the striatum, cortex, and hippocampus in control ACSF-treated animals (white bars) were 2.20 ± 0.12, 1.35 ± 0.07, and 1.05 ± 0.06 pmol/mg of tissue, respectively. Values are statistically significantly different from control values at the following levels: (*) $p < 0.05$ and (†) $p < 0.001$ ($n = 3$ for each group). Panel D shows the effects of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA on 5-HT concentrations after either intrastriatal, intracortical, or intrahippocampal drug administration (7 nmol, black bars; 20 nmol, hatched bars) four times at 12 h intervals. The absolute values for 5-HT in the striatum, cortex, and hippocampus in control ACSF-treated animals (white bars) were 3.72 ± 0.20, 1.30 ± 0.10, and 2.03 ± 0.08 pmol/mg of tissue, respectively. Values are statistically significantly different from control values at the following levels: (*) $p < 0.05$ and (†) $p < 0.005$ ($n = 3$ for each group).

**Effects of 2,5-Bis(glutathion-*S*-yl)-α-MeDA on Monoamine Neurotransmitter Levels following Intrastriatal and Intracortical Administration.** Intrastriatal administration of 2,5-bis(glutathion-*S*-yl)-α-MeDA (300 nmol) significantly ($p < 0.05$) decreased striatal 5-HT (47%) and 5-HIAA (24%) concentrations (panels A and B of Figure 2). Concentrations of 5-HT in the striatum contralateral to the site of injection were unaltered. 5-HT and 5-HIAA concentrations in the cortex ipsilateral to the site of the intrastriatal injections were also significantly ($p < 0.05$) decreased (panels A and B of Figure 2). Although hippocampal 5-HT concentrations were not affected by 2,5-bis(glutathion-*S*-yl)-α-MeDA (300 nmol), an increase in hippocampal 5-HIAA levels ipsilateral to the site of injection was found (Figure 2B). DA and NE concentrations did not change following intrastriatal administration of 2,5-bis(glutathion-*S*-yl)-α-MeDA (see the Supporting Information). Brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla) were also unaffected by the intrastriatal administration of 2,5-bis(glutathion-*S*-yl)-α-MeDA (see the Supporting Information).

Intracortical administration of 2,5-bis(glutathion-*S*-yl)-α-MeDA significantly decreased 5-HT concentrations in cortex and the striatum ipsilateral to the site of injection (Figure 2C) without affecting 5-HT levels in ipsilateral hippocampus. No changes were seen in brain 5-HIAA concentrations (see the Supporting Information). DA and NE concentrations did not change following intracortical administration of 2,5-bis(glutathion-*S*-yl)-α-MeDA (see the Supporting Information). Brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla) were also unaffected by the intracortical administration of 2,5-bis(glutathion-*S*-yl)-α-MeDA (see the Supporting Information).

**Effects of 5-(*N*-Acetylcystein-*S*-yl)-α-MeDA on Monoamine Neurotransmitter Levels following Intrastriatal, Intracortical, and Intrahippocampal Administration.** Intrastriatal administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (7 and 20 nmol) caused a decrease in striatal 5-HT and 5-HIAA concentrations only at the site of injection (Figure 2D). No effects on either 5-HT or 5-HIAA concentrations were seen in the cortex or hippocampus following intrastriatal injection of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA. DA and NE concentrations did not change following intrastriatal administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (see the Supporting Information). Brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla) were also unaffected by the intrastriatal administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (see the Supporting Information).

Intracortical administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (7 and 20 nmol) caused a decrease in cortical 5-HT and 5-HIAA concentrations only at the site of injection (Figure 2D). Concentrations of 5-HT and 5-HI-

AA in the cortex contralateral to the site of injection were unaffected. Although no significant effects on either 5-HT or 5-HIAA concentrations were seen in the striatum or hippocampus following intracortical injection of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA, 5-HT levels did decrease in the striatum. DA and NE concentrations did not change following intracortical administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (see the Supporting Information). Brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla) were also unaffected by the intracortical administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (see the Supporting Information).

Intrahippocampal administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (20 nmol) caused a decrease in hippopcampal 5-HT concentrations (Figure 2D). No effects on either 5-HT or 5-HIAA concentrations in the striatum or cortex were found. DA and NE concentrations did not change following intrahippocampal administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (see the Supporting Information). Brain regions enriched with 5-HT cell bodies (midbrain/diencephalon/telencephalon and pons/medulla) were also unaffected by the intrahippocampal administration of 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (see the Supporting Information).

## Discussion

Direct injection of either 5-(glutathion-*S*-yl)-α-MeDA (4 × 200 and 4 × 400 nmol), 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (4 × 7 and 4 × 20 nmol), or 2,5-bis(glutathion-*S*-yl)-α-MeDA (4 × 150 and 4 × 300 nmol) into the striatum, cortex, or hippocampus produces significant decreases in 5-HT concentrations 7 days after the last injection (Figures 1 and 2). In contrast, icv administration of either 5-(glutathion-*S*-yl)-α-MeDA (4 × 720 nmol) or 5-(*N*-acetylcystein-*S*-yl)-α-MeDA (4 × 100 nmol) failed to produce any detectable changes in striatal, cortical, or hippocampal 5-HT concentrations 7 days after the last injection (*24*). The inability of 5-(glutathion-*S*-yl)-α-MeDA and 5-(*N*-acetylcystein-*S*-yl)-α-MeDA to reproduce MDA-mediated serotonergic neurotoxicity following icv administration may be a consequence of the complex pharmacokinetics following icv administration, and the inherent reactivity of their metabolites which may limit their ability to reach 5-HT nerve terminal sites. However, both 5-(glutathion-*S*-yl)-α-MeDA and 5-(*N*-acetylcystein-*S*-yl)-α-MeDA produce an overt behavioral response similar to that seen following peripheral administration of MDA (*24*, *28*), indicating that these metabolites share some properties with the parent amphetamine. In addition, preliminary experiments indicate that icv administration of either 5-(glutathion-*S*-yl)-α-MeDA or 5-(*N*-acetylcystein-*S*-yl)-α-MeDA induces the activation of microglia in striatum, cortex, and hippocampus,[2] evidence that these metabolites do cause neuronal damage in these 5-HT nerve terminal-enriched sites.

The decreases in 5-HT concentrations caused by 5-(glutathion-*S*-yl)-α-MeDA, 5-(*N*-acetylcystein-*S*-yl)-α-MeDA, and 2,5-bis(glutathion-*S*-yl)-α-MeDA occur in the absence of any changes in brain DA or NE concentrations (see the Supporting Information), indicating that, like the parent amphetamine, the thioether metabolites of α-MeDA exhibit selectivity for the serotonergic system. The order of relative neurotoxic potency of the thioether metabolites of α-MeDA is as follows: 5-(*N*-acetylcystein-*S*-yl)-α-MeDA ≫ 2,5-bis(glutathion-*S*-yl)-α-MeDA > 5-(glutathion-*S*-yl)-α-MeDA, which is in accordance with their ability to produce an acute "serotonin behavioral syndrome" (*24*). The greater potency of the mercapturic acid may be due to its relative persistence in brain (*29*) and its ability to maintain redox activity by limiting intramolecular cyclization (*30*). A study using CuZn−superoxide dismutase (CuZn−SOD) transgenic mice showed that homozygous SOD transgenic mice that carry two copies of the human CuZn−SOD gene are resistant to the depletion of dopamine and DOPAC following MDMA administration (*31*), indicating an important role for ROS in the biochemical effects of MDMA. The ability of α-MeDA thioethers to redox cycle and generate ROS provides a basis for their biological reactivity, and their relative potency is likely determined by their ability to generate ROS and their persistence in the tissue.

Intrastriatal administration of either 5-(glutathion-*S*-yl)-α-MeDA or 2,5-bis(glutathion-*S*-yl)-α-MeDA caused concomitant decreases in 5-HT concentrations in the cortex ipsilateral to the site of injection, and intracortical injections produced concomitant decreases in 5-HT concentrations in the ipsilateral striatum. The basis for this "cross-talk" is not known. Two types of serotonergic axons can be distinguished in the forebrain (*32*, *33*). Thin and extensively branched fibers, with small fusiform varicosities, originate in the dorsal raphe nucleus, the so-called "D system". The "M system" consists of thick nonvaricose axons, and originates in the median raphe nucleus (*32*, *34*). The frontal cortex and striatum are both innervated primarily by axons projecting from the dorsal raphe, and 5-HT nerve terminals in these regions are vulnerable to several neurotoxic substituted amphetamines, including MDA, MDMA, and *p*-chloroamphetamine (*35*, *36*). This common origin of serotonin projections to the cortex and striatum may provide a line of communication that somehow facilitates neuronal injury to sites distal from the site of drug injection. For example, when α-MeDA thioethers are injected into the striatum or cortex, they may be transported into the serotonergic neurons via either the L-amino acid transport system which may recognize either the GSH or the cysteine moiety or the biogenic amine reuptake system which recognizes the catecholamine moiety. Therefore, TPH activity within the cell bodies, which send projections to both the striatum and cortex, may decrease as a consequence of either the redox or electrophilic properties of the polyphenolic thioether conjugates. In contrast to the adverse effects of substituted amphetamines on the fine axon endings that arise from the dorsal raphe and terminate in the cortex and striatum, they appear to spare ascending 5-HT projections from the median raphe (*35*, *36*). This may explain why direct injections into the striatum and cortex fail to produce significant serotonergic neurotoxicity in the hippocampus, which exclusively receives projections from the median raphe.

A single dose of MDMA (20 mg/kg) decreases cortical and hippocampal 5-HT concentrations to about 30−70% of control levels 1 week after drug administration (*37*). The extents of 5-HT and 5-HIAA decreases also vary in a broad range because of the different dosing regimens used by individual researchers (*11*, *12*, *38*). 5-(*N*-Acetylcystein-*S*-yl)-α-MeDA depletes brain 5-HT and 5-HIAA

[2] X. Li et al., unpublished observations.

concentrations at a much lower dose than either 5-(glutathion-*S*-yl)-α-MeDA or 2,5-bis(glutathion-*S*-yl)-α-MeDA. In addition, the serotonin deficits caused by MDA and MDMA can last for months (*12*). The time at which the neurotoxic effects of α-MeDA thioethers reach a maximum is not known and will require further experimentation. It is possible that the toxicity may still be progressing 1 week after termination of the treatment. Doses of the α-MeDA thioethers required to produce serotonergic neurotoxicity are much lower (0.03−1.72%) than doses of MDA producing a similar degree of toxicity, even accounting for differences in the route of administration. Moreover, the doses used in this study likely fall within the range of α-MeDA thioethers levels present in the brain following MDA (93 μmol/kg, sc) administration (*27*). The mechanism(s) by which α-MeDA thioethers produce deficits in 5-HT and 5-HIAA concentrations is not known. However, the finding that α-MeDA fails to reproduce the neurobehavioral and neurotoxicological effects of MDA suggests that the effects of α-MeDA thioethers are not solely dependent upon the catecholamine moiety.

Systemic metabolism of MDMA and MDA seems to play an important role in their neurotoxicity. MDMA undergoes N-demethylation to form MDA, and both MDMA and MDA are demethylenated (*21*, *22*), a reaction which generates the corresponding catechols (*N*-methyl-α-methyldopamine and α-methyldopamine, respectively) and which is catalyzed by P450 2B, P450 2D, and P450 3A (*23*). Potential differences in the activity of these enzymes may therefore play an important role in predisposing certain individuals to the adverse effects of MDA and MDMA. However, neither the mechanism nor the enzymes involved in the oxidation of the catechols, nor their thioether conjugates, are known. Variability in this oxidation would have important consequences in terms of susceptibility to neurotoxicity. Conjugation of α-MeDA, or rather the *o*-quinone, to GSH occurs nonenzymatically, but it is also likely catalyzed by GSH *S*-transferase isoforms. Although it is not known which isoforms are involved, perhaps the μ class isoforms are particularly important, since GSH *S*-transferase M2-2 has been shown to participate in the very specific conjugation of the DA metabolite aminochrome to GSH (*39*, *40*). Although these authors have postulated that the conjugation of the quinone metabolites of dopamine with GSH constitutes a detoxication reaction, the reactivity of the corresponding cysteine and *N*-acetylcysteine metabolites is well established (*41*−*46*). For example, 5-*S*-cysteinyl-3,4-dihydroxyphenylacetate is specifically cytotoxic to differentiated P19 neuroglial cell cultures and pyramidal neurons in organotypic cultures of hippocampus (*41*), and Dryhurst and colleagues have elegantly demonstrated the neurotoxic potential of several metabolites formed following the conjugation of dopamine *o*-quinone with cysteine (*42*−*46*). The potential of these metabolites to cause neurotoxicity in the brain in vivo will depend on the complex factors that govern the distribution and elimination of such hydrophilic molecules (*47*). The ability of the mercapturic acid pathway to modulate the redox properties of quinol thioethers (*48*) will also contribute to the relative reactivity of each of the metabolites along this pathway.

Variations in the transport of the conjugates into the brain will also be an important determinant of susceptibility, and we know very little about human variability in γ-glutamyl transpeptidase at the blood−brain barrier, and virtually nothing about the activity of the intact GSH transporter at the blood−brain barrier. The ratio of N-acetylation to N-deacetylation of the thioether metabolites is also very important, because maintaining the conjugate in the N-acetylated form (i.e., as the mercapturic acid) may result in persistence of the metabolite in brain, and retention of the redox activity of the conjugate (*28*). Variability in DA and 5-$HT_2$ receptor number and activity may be an important determinant of the response to MDA and MDMA, because DA receptors (*49*) and 5-$HT_2$ receptors (*50*, *51*) may play important roles in the development of the serotonergic neurotoxicity. Finally, variability in antioxidant defenses may also predispose certain individuals to the neurotoxic response. A variety of different factors may therefore predispose certain individuals within the population to the potential adverse effects of these amphetamine analogues.

In summary, we have shown that three thioether metabolites of α-MeDA, 5-(*N*-acetylcystein-*S*-yl)-α-MeDA, 5-(glutathion-*S*-yl)-α-MeDA, and 2,5-bis(glutathion-*S*-yl)-α-MeDA, produce serotonergic neurotoxicity when injected directly into regions of the brain known to be susceptible to MDA- and MDMA-mediated toxicity. In addition, each metabolite demonstrates selectivity for the serotonergic system, in that neither the DA nor the NE neurotransmitter systems are affected by these metabolites (see the Supporting Information). Such selectivity is a hallmark of MDA- and MDMA-mediated neurotoxicity in the rat. Nonetheless, a role for thioether conjugates of α-MeDA in the toxicity observed following systemic administration of MDA and MDMA remains to be demonstrated, and requires further experimentation.

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**Supporting Information Available:** Tables 1-4 show the lack of an effect of MDA, 5-(*N*-acetylcystein-*S*-yl)-α-MeDA, 5-(glutathion-*S*-yl)-α-MeDA, and 2,5-bis(glutathion-*S*-yl)-α-MeDA, on DA and NE concentrations in the cortex, striatum, and hippocampus, and Tables 5−8 show the lack of effect of MDA, 5-(*N*-acetylcystein-*S*-yl)-α-MeDA, 5-(glutathion-*S*-yl)-α-MeDA, and 2,5-bis(glutathion-*S*-yl)-α-MeDA, on 5-HT and 5-HIAA concentrations in midbrain/diencephalon/telencephalon and pons/medulla. This material is available free of charge via the Internet at http://pubs.acs.org.

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