

# Effects of Intracerebroventricular Administration of 5-(Glutathion-S-yl)- $\alpha$ -methyldopamine on Brain Dopamine, Serotonin, and Norepinephrine Concentrations in Male Sprague-Dawley Rats<sup>†</sup>

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$\alpha$ -Methyldopamine ( $\alpha$ -MeDA) is a metabolite of the serotonergic neurotoxicants 3,4-( $\pm$ )-(methylenedioxy)amphetamine (MDA) and 3,4-( $\pm$ )-(methylenedioxy)methamphetamine (MDMA).  $\alpha$ -MeDA readily oxidizes, and in the presence of glutathione (GSH) it forms 5-(glutathion-S-yl)- $\alpha$ -methyldopamine [5-(glutathion-S-yl)- $\alpha$ -MeDA]. Since GSH conjugates of many polyphenols are biologically (re)active, we investigated the role of 5-(glutathion-S-yl)- $\alpha$ -MeDA in the acute and long-term neurochemical changes observed after administration of MDA. Intracerebroventricular (icv) administration of 5-(glutathion-S-yl)- $\alpha$ -MeDA (720 nmol) to male Sprague-Dawley rats produced behavioral changes similar to those reported after subcutaneous administration of MDA. Thus, animals became hyperactive and aggressive and displayed forepaw treading and Straub tails, behaviors usually seen after administration of serotonin (5-HT) releasers, and consistent with a role for 5-(glutathion-S-yl)- $\alpha$ -MeDA in some of the behavioral alterations seen after administration of MDA and MDMA. In addition to the behavioral changes, 5-(glutathion-S-yl)- $\alpha$ -MeDA also caused short-term alterations in the dopaminergic, serotonergic, and noradrenergic systems. An increase in dopamine synthesis appears to be a prerequisite for the long-term depletion of brain 5-HT following MDMA administration. However, although 5-(glutathion-S-yl)- $\alpha$ -MeDA reproduced some of the effects of MDA on the dopaminergic system and was capable of causing acute increases in 5-HT turnover, a single icv injection of 5-(glutathion-S-yl)- $\alpha$ -MeDA did not result in long-term serotonergic toxicity. Thus, although acute stimulation of dopamine turnover may be necessary for long-term serotonergic toxicity, such changes are not sufficient to produce these effects. The effects of a multiple dosing schedule of 5-(glutathion-S-yl)- $\alpha$ -MeDA will therefore require investigation before we can define a role for this metabolite in MDA and MDMA mediated neurotoxicity. MDA also produces a pressor response that is related to its ability to release neuronal norepinephrine stores, and 5-(glutathion-S-yl)- $\alpha$ -MeDA caused comparable depletions of brain norepinephrine concentrations, indicating that both compounds produce similar effects on the noradrenergic system.

## Introduction

3,4-( $\pm$ )-(Methylenedioxy)amphetamine (MDA) and 3,4-( $\pm$ )-(methylenedioxy)methamphetamine (MDMA) are ring-substituted amphetamine derivatives, structurally related to psychomotor stimulant amphetamines and the hallucinogen mescaline. In recent years, their clandestine manufacture and appearance on the street have made them popular drugs of abuse (1, 2) for their ability to induce "a state of sensory amplification and enhance-

ment without appreciable sympathomimetic stimulation" (3), and they have been reported as useful adjuncts to psychotherapy (4). After misuse, chronic paranoid psychosis has been reported, which is persistent and resistant to treatment with haloperidol (5). The actions of MDA and MDMA are biphasic, initially causing an acute release of 5-hydroxytryptamine (5-HT) (6) followed by prolonged depletion of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) (7, 8) and structural damage to 5-HT terminal and preterminal axons in various regions of the central nervous system (7, 9). The immediate 5-HT release caused by these compounds can be blocked *in vitro* by 5-HT uptake inhibitors (10). The long-term neurotoxicity can also be blocked *in vivo* by 5-HT uptake inhibitors (11) and by 5-HT receptor antagonists, but is potentiated by L-dopa (12). The neurotoxic effects of MDA and MDMA are dependent on the route and frequency of drug administration (13). Direct injection of either MDA or MDMA into the brain fails to reproduce the neurotoxicity following peripheral administration, indicating that the parent amphetamines are unlikely to be responsible for the neurotoxic effects (14) and suggesting that systemic metabolism plays an important

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<sup>1</sup> Abbreviations: 3,4-( $\pm$ )-(methylenedioxy)amphetamine [MDA]; 3,4-( $\pm$ )-(methylenedioxy)methamphetamine [MDMA]; glutathione [GSH];  $\gamma$ -glutamyl transpeptidase [ $\gamma$ -GT];  $\alpha$ -methyldopamine [ $\alpha$ -MeDA]; dihydroxyphenylacetic acid [DOPAC]; homovanillic acid [HVA]; serotonin [5-HT]; 5-hydroxyindole acetic acid [5-HIAA]; intracerebroventricular [icv]; brain uptake index [BUI].

role in the development of toxicity. Indeed, the finding that MDMA does not affect the serotonergic system in mice has been attributed to metabolic differences between the two species (15).

Several known and putative metabolites of MDA and MDMA fail to induce monoaminergic neurotoxicity following intracerebroventricular (icv) administration, including 3,4-dihydroxymethamphetamine (16) and 2-hydroxy-4,5-MDMA (17). Although the 6-hydroxydopamine analog, 2,4,5-trihydroxymethamphetamine, caused depletion of both dopamine and serotonin following intrastriatal and intracortical administration to rats (16, 17), mechanisms for its uptake across the blood-brain barrier remain to be established. Both MDA and MDMA are metabolized to  $\alpha$ -methyldopamine ( $\alpha$ -MeDA) *in vitro* (18, 19) and *in vivo* (20, 21). Rat liver and brain cytochrome P-450 isozymes catalyze the demethylenation of MDMA to the corresponding catechol (18, 22). The demethylenation of MDMA can also be catalyzed by hydroxyl radical (23). However, intracerebral injection of either 3-O-methyl- $\alpha$ -MeDA or  $\alpha$ -MeDA also fails to induce monoaminergic neurotoxicity (24). Thus the nature of the metabolite(s) responsible for MDA- and MDMA-mediated neurotoxicity remains unclear.

$\alpha$ -MeDA and *N*-methyl- $\alpha$ -MeDA are readily oxidized by superoxide anion ( $O_2^{\cdot-}$ ) to the *o*-quinones, which can react with both protein and nonprotein sulfhydryls, including glutathione (GSH) (22, 25). Conjugation of polyphenols with GSH frequently results in either preservation of, or enhancement of biological (re)activity (26). For example, quinone thioethers retain the ability to redox cycle and produce reactive oxygen species (27) and to arylate tissue macromolecules (28). In addition, quinone thioethers have been shown to be substrates for, or inhibitors of enzymes which utilize GSH and/or quinones as substrates (26). Therefore, because (i) neither MDA nor MDMA produce neurotoxicity when injected directly into brain, (ii) icv administration of some major metabolites of MDA fails to reproduce the neurotoxicity, (iii)  $\alpha$ -MeDA is a metabolite of both MDA and MDMA, (iv)  $\alpha$ -MeDA is readily oxidized to the corresponding quinone which can undergo conjugation with GSH, and (v) quinone thioethers exhibit a variety of toxicological activities, we investigated the potential role of thioether metabolites of  $\alpha$ -MeDA in the neurotoxicity of MDA and MDMA. We report that icv administration of 5-(glutathion-S-yl)- $\alpha$ -MeDA to male Sprague-Dawley rats causes behavioral changes identical to those observed following subcutaneous administration of MDA. In addition, 5-(glutathion-S-yl)- $\alpha$ -MeDA caused acute changes in the dopaminergic, serotonergic, and noradrenergic neurotransmitter systems similar to those caused by MDA. The results are discussed with respect to the potential contribution of thioether metabolites to the neurotoxicity of MDA and MDMA.

## Materials and Methods

**Chemicals.** Reduced glutathione and mushroom tyrosinase (5600 U/mg) were obtained from Sigma Chemical Co. (St. Louis, MO).  $\alpha$ -MeDA was a generous gift from Dr. Anthony Lu at Merck Research Laboratories (Rahway, NJ). MDA was supplied by the Research Technology Branch, National Institute on Drug Abuse, Rockville, MD. [ $^3H$ ]( $\pm$ )-MDA was provided by NIDA (0.59 Ci/mmol). 5-(Glutathion-S-yl)- $\alpha$ -MeDA was synthesized according to previously published methods (24) with modifications (29) and the structure confirmed by  $^1H$ -NMR. 5-(Glutathion-S-yl)-[ $^3H$ ]- $\alpha$ -MeDA was synthesized by standard proce-

dures. Briefly, [ $^3H$ ]( $\pm$ )-MDA was demethylenated, to yield  $\alpha$ -[ $^3H$ ]-MeDA, with a 2-fold molar excess of boron trichloride in methylene chloride, under argon. [ $^3H$ ]( $\pm$ )- $\alpha$ -MeDA was then used to prepare 5-(glutathion-S-yl)-[ $^3H$ ]- $\alpha$ -MeDA as described (29). All other chemicals were of the highest grade commercially available.

**Animals.** Male Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX; 200–225 g) were used in all experiments. The rats were maintained on a 12 h light/dark cycle and were allowed free access to food and water before and during the experiment.

**Effects of MDA and 5-(Glutathion-S-yl)- $\alpha$ -methyldopamine on Neurotransmitter Levels.** Surgery was performed to place a guide cannula in the left lateral ventricle of male rats to facilitate icv injections. Rats were anesthetized with 3.5 mL/kg of a mixture containing chloral hydrate (37.5 mg/mL) and sodium pentobarbital (9.4 mg/mL), and heads were shaved and placed in a stereotaxic apparatus. A mid-sagittal incision was made with a surgical scalpel in order to expose the skull. The skull was leveled and the nosebar raised 5 mm above the intraural line. A small burr hole was made with a hand drill: (–) 0.6 mm from bregma, (–) 2.0 mm lateral to the midline. A 26 g guide cannula (Plastics One, Roanoke, VA) was lowered 3.2 mm ventral to the surface of the skull in order to guide an injection needle which extends 1.5 mm below the bottom of the guide cannula. Three other burr holes were made for jeweler's screws. Cranioplastic (Plastics One, Roanoke, VA) 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, and the incision was covered with a thin layer of triple antibiotic ointment (Neosporin, Burroughs Wellcome Co.). The incision was closed with sutures, and the animals were allowed to recover for 5–7 days. At the end of this period, the animals were dosed using 10  $\mu$ L of artificial cerebrospinal fluid (147 mM NaCl, 4 mM KCl, 1.2 mM  $CaCl_2$ , and 1.2 mM  $MgSO_4$ ) as the vehicle. 5-(Glutathion-S-yl)- $\alpha$ -MeDA was infused into the left lateral ventricle of the awake animals at a rate of 2  $\mu$ L every 30 s (total volume 10  $\mu$ L) by using a Hamilton syringe connected to an injection needle. The injection needle was left in place for a period of 1–2 min after the injection. A dummy cannula was then inserted into the guide cannula to close the injection site. Control animals received 10  $\mu$ L of artificial cerebrospinal fluid (icv). Since 5-(glutathion-S-yl)- $\alpha$ -MeDA is a metabolite of MDA and because the effects of MDA on the brain are reasonably well documented, MDA was employed as the positive control. Following peripheral administration of MDA (23  $\mu$ mol (20 mg/kg); sc) or icv administration of 5-(glutathion-S-yl)- $\alpha$ -MeDA (720 nmol (3.1% of the dose of MDA)) animals were euthanized by decapitation at 0.5, 1, 2, 6, and 168 h and brains quickly removed onto a chilled plate. The occipital cortex, hippocampus, striatum, hypothalamus, the area of the brainstem corresponding to the pons/medulla, and the midbrain/diencephalon/telencephalon were dissected free, immediately placed in preweighed amber microcentrifuge tubes, and placed into liquid nitrogen. Brains were dissected in this manner in order to obtain regions which are nerve terminal fields and major targets for MDA- and MDMA-mediated neurotoxicity (cortex, hippocampus, striatum, hypothalamus) and also regions which contain nerve cell bodies (midbrain/diencephalon/telencephalon, pons/medulla). The tissue samples were then stored at  $-70^\circ C$  until assayed (within 1 week). Tissue samples were prepared for analysis by reweighing the microcentrifuge tubes, calculating tissue weights, and sonicating the tissue in ice-cold 0.1 N  $HClO_4$  containing 134  $\mu$ M EDTA and 263  $\mu$ M  $Na_2S_2O_5$  for 30 s with a cell disrupter. The sonicated tissue was then centrifuged at 13500g for 10 min in a table-top microcentrifuge (Eppendorf; Model 5415c). Supernatants were then filtered through 0.22  $\mu$ m nylon filters (Acrodisc-13, Gelman Sciences), and 20  $\mu$ L of the filtrate was used for HPLC analysis.

**HPLC-CEAS Neurotransmitter Analysis.** Norepinephrine, dopamine, DOPAC, HVA, 5-HT, and 5-HIAA levels were determined simultaneously by HPLC equipped with an 8 channel coulometric electrode array system (HPLC-CEAS; ESA

Inc., Chelmsford, MA). The potential applied to the first electrode was 0 mV and was increased in increments of 50 mV to a final potential of +350 mV. Data from the hydrodynamic behavior of each analyte aid in the confirmation of peak identity and purity. The mobile phase consisted of 4 mM citrate, 8 mM ammonium acetate, 54  $\mu$ M EDTA, 230  $\mu$ M 1-octanesulfonic acid (Eastman Kodak Co., Rochester, NY), and 5% methanol, pH 2.5. Separation was accomplished by use of an ESA HR-80 column (80  $\times$  4.6 mm i.d.; 3  $\mu$ m particle size). The flow rate was held constant at 1 mL/min. Retention times (in parentheses) for the compounds were as follows: norepinephrine (2.5 min), dopamine (5.5 min), DOPAC (4.25 min), HVA (19.5 min), 5-HT (18.0 min), and 5-HIAA (12.25 min). Quantitation of neurotransmitters and their metabolites was accomplished by comparison of peak areas of samples with standard curves generated from authentic standards during each series of HPLC analyses. Data are expressed as ng/mg or pg/mg tissue (wet weight) and were analyzed by ANOVA followed by a *post hoc* Student Newman-Kuels test, or by the Student *t*-test.

**Brain Uptake Measurements.** Brain uptake indices (BUI) were determined by the method of Oldendorf (30). This method allows for the measurement of uptake by animal brain of [ $^3$ H]-, [ $^{35}$ S]-, or [ $^{14}$ C]-labeled substances. A mixture of 5-(glutathion-S-yl)-[ $^3$ H]- $\alpha$ -MeDA and [ $^{14}$ C]-1-butanol, with or without competing substrate or inhibitors, containing approximately 1  $\mu$ Ci of each radionuclide in 0.2 mL Krebs–Ringer buffer, was prepared in a glass tuberculin syringe. [ $^{14}$ C]Butanol has been shown to be 100% extracted by the brain during a single passage (31). Male Sprague-Dawley rats (mean weight 273 g) were anesthetized with sodium pentobarbital (50 mg/kg, ip). The rats were positioned supine, and the anterior neck skin was incised. Following blunt dissection of the fascia tissue and neck muscles, the left common carotid artery was exposed. The carotid was punctured with a 30 g needle, and 0.2 mL of the radioactive mixture was injected over a period of approximately 0.25 s. The injection is made at a rate which is sufficiently high to minimize mixing with rat plasma. This allows isolated exposure of 5-(glutathion-S-yl)-[ $^3$ H]- $\alpha$ -MeDA to blood–brain barrier carrier sites without competition from substances in free solution in plasma. The needle was left in place, and 15 s after injection the animals were decapitated with a guillotine. In some experiments, 5-(glutathion-S-yl)-[ $^3$ H]- $\alpha$ -MeDA was coinjected with 1 mM GSH, or rats were pretreated with acivicin (AT-125; 18 mg/kg) 20 min prior to the injection of 5-(glutathion-S-yl)-[ $^3$ H]- $\alpha$ -MeDA and [ $^{14}$ C]butanol. The brains were removed, minced with scissors, and homogenized in 5 mL of water, and the resulting homogenate was digested overnight following the addition of 1 mL of 10 N NaOH. Following digestion, 100  $\mu$ L of 30% hydrogen peroxide was added to 200  $\mu$ L of sample and incubated at room temperature for 10 min to decolorize the sample. Following decolorization, 100  $\mu$ L of glacial acetic acid was added and radioactivity in the mixture determined by dual label liquid scintillation spectroscopy. An aliquot of the original isotope mixture was counted at the same time. The ratio of [ $^{14}$ C] to [ $^3$ H] in brain relative to the ratio of [ $^{14}$ C] to [ $^3$ H] in the original mixture determines the amount of 5-(glutathion-S-yl)-[ $^3$ H]- $\alpha$ -

**Table 1. Behavioral Profile following Administration of MDA,  $\alpha$ -MeDA, or 5-(Glutathion-S-yl)- $\alpha$ -MeDA<sup>a</sup>**

|                                            | hyper-activity | forepaw treading | Straub tail | low posture |
|--------------------------------------------|----------------|------------------|-------------|-------------|
| MDA (sc) (93 $\mu$ mol/kg)                 | yes            | yes              | yes         | yes         |
| $\alpha$ -MeDA (icv) (2.4 and 3 $\mu$ mol) | no             | no               | no          | no          |
| 5-(GSyl)- $\alpha$ -MeDA (icv) (720 nmol)  | yes            | yes              | yes         | yes         |

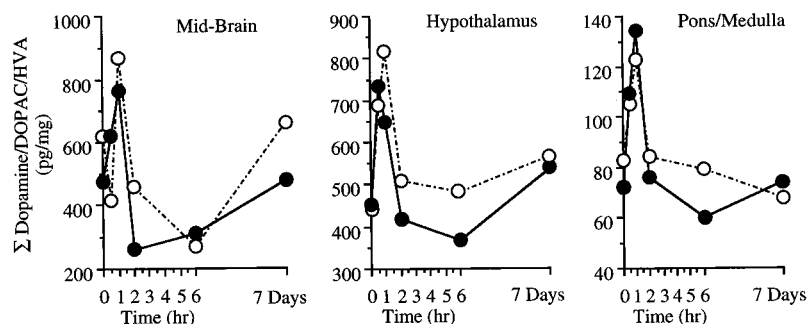
<sup>a</sup> Behaviors were observed in all treated animals ( $n = 20$ –28 for MDA and 5-(GSyl)- $\alpha$ -MeDA;  $n = 3$  for  $\alpha$ -MeDA).

MeDA lost to brain tissue on a single passage through the brain microcirculation.

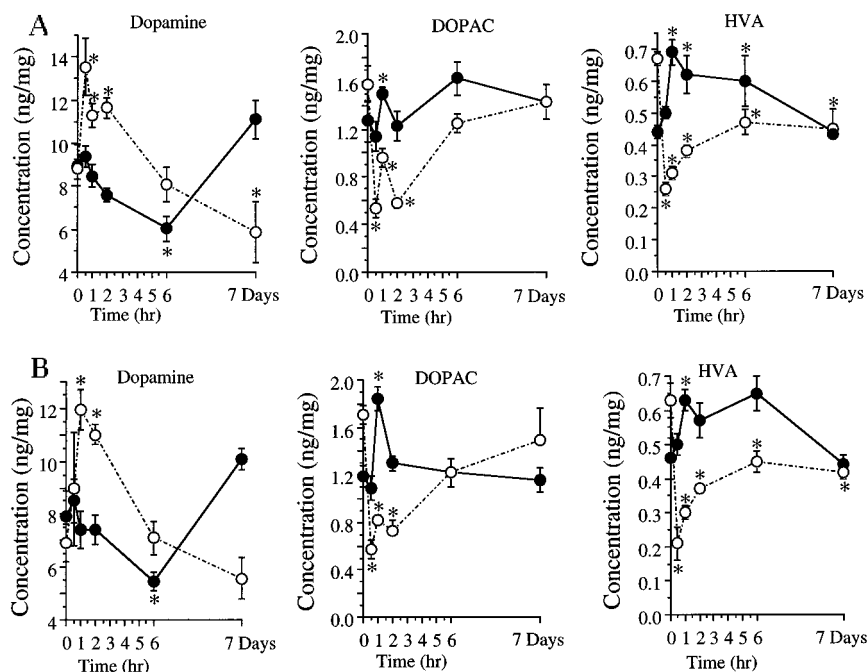
## Results

**Behavioral Effects of  $\alpha$ -Methyldopamine and 5-(Glutathion-S-yl)- $\alpha$ -methyldopamine.** Following icv administration of  $\alpha$ -MeDA (2.4 and 3.0  $\mu$ mol) and 5-(glutathion-S-yl)- $\alpha$ -MeDA (720 nmol), behavior, weight, and food and water intake were monitored (doses  $> 720$  nmol of 5-(glutathion-S-yl)- $\alpha$ -MeDA were lethal). Differing behavioral effects were observed between treatment groups (Table 1). Behaviors appeared rapidly (1–2 min) following icv administration of 5-(glutathion-S-yl)- $\alpha$ -MeDA and  $\alpha$ -MeDA and peaked in intensity during the first 30 min. In contrast, animals receiving MDA subcutaneously exhibited a more gradual change in behaviors approximately 15 min after drug administration, perhaps indicative of a requirement for absorption, metabolism, and distribution processes.  $\alpha$ -MeDA-treated animals became docile; 5-(glutathion-S-yl)- $\alpha$ -MeDA-treated animals became hyperactive, aggressive, and displayed forepaw treading and Straub tails, all of which are consistent with a role for 5-(glutathion-S-yl)- $\alpha$ -MeDA in some of the behavioral alterations seen after administration of 5-HT-releasers such as MDA and MDMA. We therefore asked the following question: “Does 5-(glutathion-S-yl)- $\alpha$ -MeDA cause changes in neurotransmitter levels in a manner similar to that of MDA?”

**Effects of MDA and 5-(Glutathion-S-yl)- $\alpha$ -methyldopamine on Brain Neurotransmitter Systems. (A) Dopaminergic Effects.** In addition to long-term neurotoxic effects on the serotonergic system, MDA and MDMA also cause the acute release of 5-HT, dopamine, and norepinephrine (32, 33). This property appears to be of functional significance. An increase in dopamine synthesis appears to be a prerequisite for the long-term depletion of brain 5-HT following MDMA administration (12). We therefore investigated the effects of MDA and 5-(glutathion-S-yl)- $\alpha$ -MeDA on the brain dopamine sys-



**Figure 1.** Increased dopamine turnover as measured by the sum ( $\Sigma$ ) of dopamine, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in midbrain/diencephalon/teiencephalon, hypothalamus, and pons/medulla following administration of (○) MDA (93  $\mu$ mol/kg, sc) or (●) 5-(glutathion-S-yl)- $\alpha$ -MeDA (720 nmol, icv). Dopamine, DOPAC, and HVA concentrations were determined by HPLC-CEAS as described in Materials and Methods. Each data point represents the mean value of 5–7 animals.



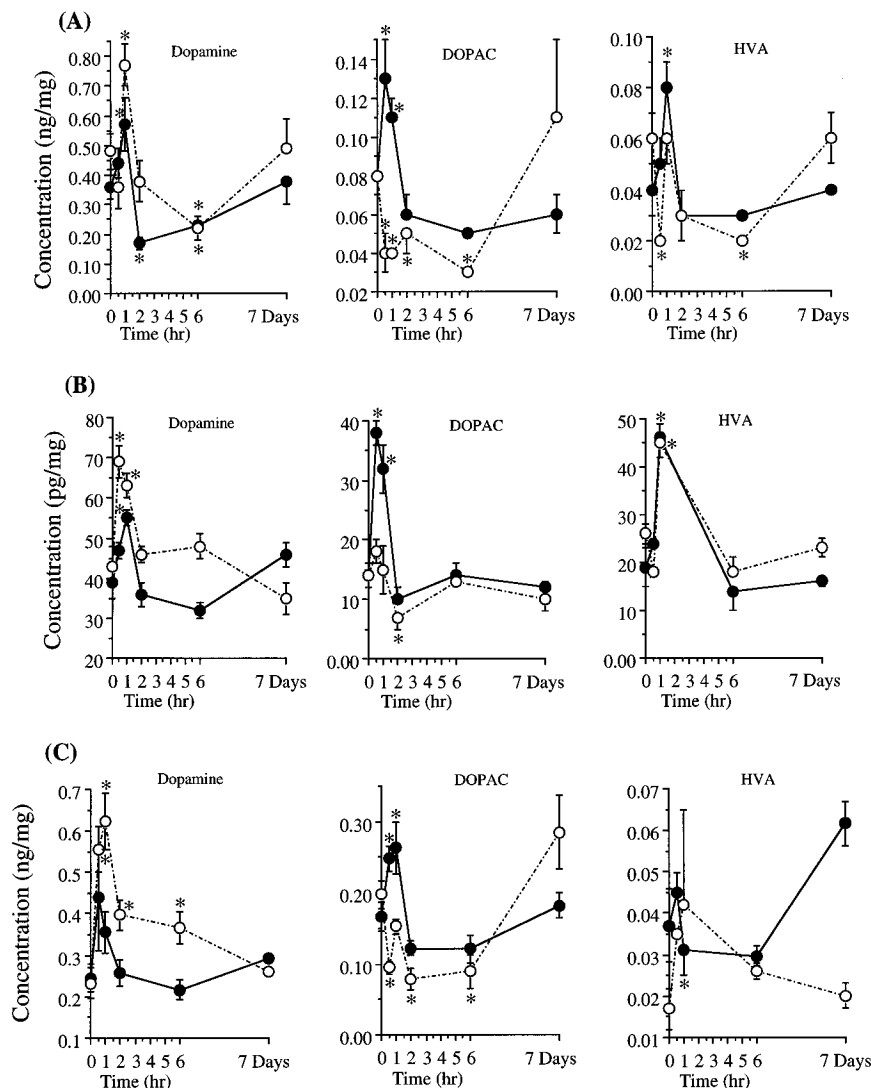
**Figure 2.** Effect of (○) MDA (93  $\mu$ mol/kg, sc) or (●) 5-(glutathion-S-yl)- $\alpha$ -MeDA (720 nmol, icv) on dopamine, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) concentrations, in striatum ipsilateral (A) and contralateral (B) to the site of intracerebroventricular injection. Dopamine, DOPAC, and HVA concentrations were determined by HPLC-CEAS as described in Materials and Methods. Each data point represents the mean  $\pm$  SE of 5–7 animals. \* Values are significantly different from respective controls at  $p < 0.05$ .

tem (Figures 1–3). Both MDA and 5-(glutathion-S-yl)- $\alpha$ -MeDA caused an increased turnover of brain dopamine. The effects of both compounds on dopamine levels in the midbrain/diencephalon/telencephalon, hypothalamus, and pons/medulla were remarkably similar (Figure 1). MDA also increased striatal dopamine concentrations by 74% and 54% (ipsilateral and contralateral, respectively; Figure 2, panels A and B). The pattern of changes caused by 5-(glutathion-S-yl)- $\alpha$ -MeDA was similar in striatal tissue either ipsilateral or contralateral to the site of injection. In contrast to MDA, 5-(glutathion-S-yl)- $\alpha$ -MeDA had little effect on striatal dopamine levels, but increased the concentration of dopamine metabolites between 28% and 57%, indicative of an ability to increase dopamine turnover. A single icv injection of 5-(glutathion-S-yl)- $\alpha$ -MeDA, at a dose 32-fold lower than that of MDA, mimicked some of the acute dopaminergic effects of the parent compound.

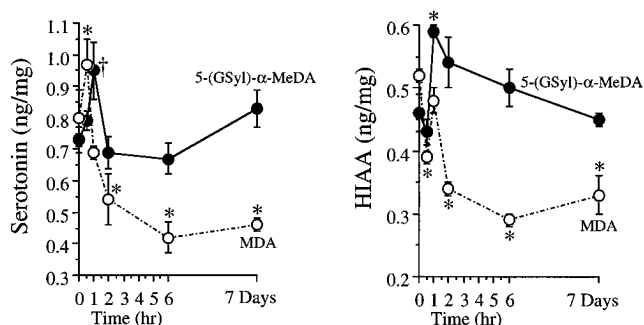
MDA increased dopamine concentrations in all areas of the brain examined. However, elevations in brain dopamine concentrations by MDA were not accompanied by corresponding increases in DOPAC (Figures 2 and 3), the MAO-catalyzed metabolite of dopamine. Rather, significant decreases in DOPAC occurred. This has previously been interpreted to be due to a decrease in intraneuronal cytosolic dopamine concentrations rather than MAO inhibition (34). Thus, MDA, akin to amphetamine, deprives MAO of its substrate with a subsequent decrease in the formation of DOPAC. In contrast to MDA, 5-(glutathion-S-yl)- $\alpha$ -MeDA caused a rapid and significant increase in brain DOPAC (Figures 2 and 3), perhaps because 5-(glutathion-S-yl)- $\alpha$ -MeDA prevents vesicular dopamine uptake. The effects of MDA and 5-(glutathion-S-yl)- $\alpha$ -MeDA on HVA concentrations also varied and were dependent upon the brain region examined. HVA concentrations initially declined following MDA administration, but, in contrast to DOPAC, eventually returned to control values. This relationship is

identical to the effects of amphetamine on dopamine metabolism. However, the kinetics of the rebound in HVA concentrations differed between regions. In contrast to MDA, 5-(glutathion-S-yl)- $\alpha$ -MeDA did not cause an observable decrease in HVA concentrations. However, it is possible that 5-(glutathion-S-yl)- $\alpha$ -MeDA caused a rapid decline in HVA that was not detected because the initial sampling time was 30 min post-injection. The earlier onset of the behavioral effects following 5-(glutathion-S-yl)- $\alpha$ -MeDA (see above) would be consistent with this possibility.

**(B) Serotonergic Effects.** Administration of MDA to male rats caused long-term depletion in brain 5-HT concentrations (Figures 4 and 5) consistent with previously published studies (7, 13). The serotonergic neurotoxicity was preceded by the rapid elevation of brain 5-HT concentrations. Thus, the reported effects of MDA on both the dopaminergic and serotonergic systems were reproduced in the present study. 5-(Glutathion-S-yl)- $\alpha$ -MeDA also increased turnover of 5-HT, as evidenced by increases in 5-HT concentrations in the midbrain/diencephalon/telencephalon (Figure 4) and by increases in 5-HIAA in the midbrain/diencephalon/telencephalon (Figure 4), cortex, hippocampus, and striatum (Figure 5). The pattern of changes caused by 5-(glutathion-S-yl)- $\alpha$ -MeDA were similar in tissue taken either ipsilateral or contralateral to the site of injection (Figures 4 and 5). However, a single icv injection of 5-(glutathion-S-yl)- $\alpha$ -MeDA did not produce long-term deficits in the serotonergic system. Thus, although 5-(glutathion-S-yl)- $\alpha$ -MeDA mimicked some of the effects of MDA on the dopaminergic system, and was capable of causing acute increases in 5-HT turnover, the current protocol did not result in long-term serotonergic toxicity. The effects of a multiple dosing schedule of 5-(glutathion-S-yl)- $\alpha$ -MeDA will therefore require investigation before we can define a role for this metabolite in MDA- and MDMA-mediated neurotoxicity.



**Figure 3.** Effect of (○) MDA (93  $\mu$ mol/kg, sc) or (●) 5-(glutathion-S-yl)- $\alpha$ -MeDA (720 nmol, icv) on dopamine, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) concentrations in midbrain/diencephalon/telencephalon (A), pons/medulla (B), and hypothalamus (C). Dopamine, DOPAC, and HVA concentrations were determined by HPLC-CEAS as described in Materials and Methods. Each data point represents the mean  $\pm$  SE of 5–7 animals. \* Values are significantly different from respective controls at  $p < 0.05$ .

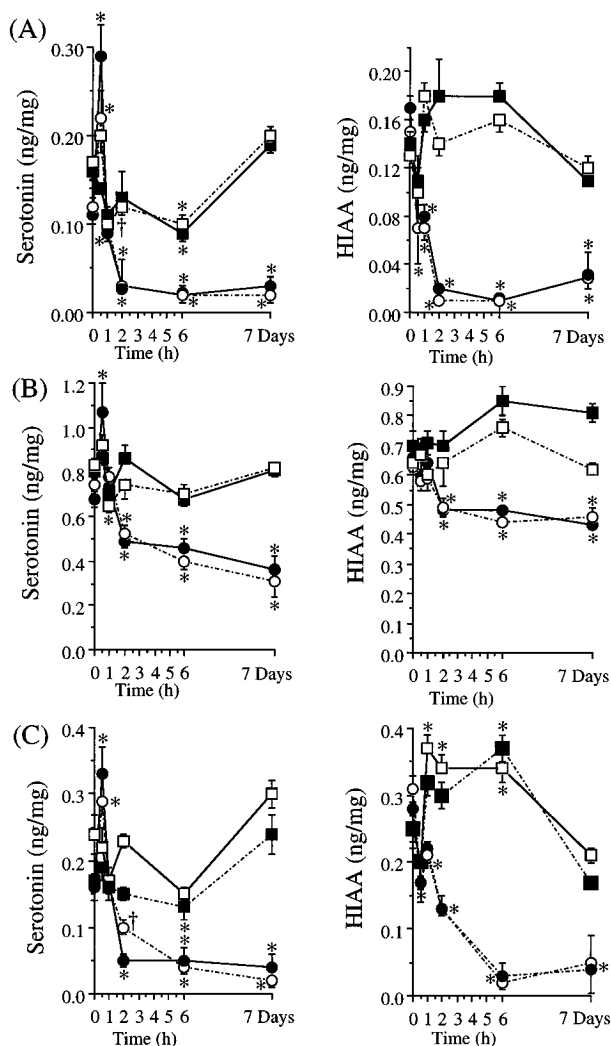


**Figure 4.** Effect of (○) MDA (93  $\mu$ mol/kg, sc) or (●) 5-(glutathion-S-yl)- $\alpha$ -MeDA (720 nmol, icv) on serotonin and 5-hydroxyindole acetic acid (5-HIAA) concentrations in midbrain/diencephalon/telencephalon. Serotonin and HIAA concentrations were determined by HPLC-CEAS as described in Materials and Methods. Each data point represents the mean  $\pm$  SE of 5–7 animals. \* Values are significantly different from respective controls at  $p < 0.05$ , †  $p > 0.05$  but  $< 0.06$ .

**(C) Stimulatory Effects.** MDA is also known to produce a pressor response that is related to its ability to release neuronal norepinephrine stores (35). Administration of MDA or 5-(glutathion-S-yl)- $\alpha$ -MeDA caused

parallel, time-dependent depletion of brain norepinephrine concentrations (Figure 6). The initial elevations in brain norepinephrine after MDA, which precede the depletion, are not observed following 5-(glutathion-S-yl)- $\alpha$ -MeDA. This may be a kinetic effect, in that our earliest sampling time (30 min) following icv administration of 5-(glutathion-S-yl)- $\alpha$ -MeDA may miss the narrow time window of elevated norepinephrine concentrations. Again, the behavioral observations may be consistent with this scenario. However, the parallel in the norepinephrine profiles, beyond 30 min following MDA and 5-(glutathion-S-yl)- $\alpha$ -MeDA, indicates both compounds produce similar effects on the noradrenergic system.

**Brain Uptake of 5-(Glutathion-S-yl)- $\alpha$ -methyl-dopamine.** Our hypothesis that quinone thioether metabolites play an important role in MDMA- and MDA-mediated neurotoxicity is dependent upon the ability of systemically synthesized metabolites to be transported across the blood–brain barrier and gain access to the neuronal compartment. The BUI for 5-(glutathion-S-yl)-[ $^3$ H]- $\alpha$ -MeDA (8.5  $\mu$ M) was  $7.4 \pm 0.5\%$  (mean  $\pm$  SD,  $n = 5$ ; Figure 7). Pretreatment of animals with acivicin (AT-125, 18 mg/kg) 20 min prior to injection of 5-(glutathion-S-yl)-[ $^3$ H]- $\alpha$ -MeDA caused a substantial increase in the



**Figure 5.** Effect of (○, ●) MDA (93 μmol/kg, sc) or (□, ■) 5-(glutathion-S-yl)-α-MeDA (720 nmol, icv) on serotonin and 5-hydroxyindole acetic acid (5-HIAA) concentrations in cortex (A), striatum (B), and hippocampus (C). Samples were taken ipsilateral (●, ■) or contralateral (○, □) to the site of intracerebroventricular injection. Serotonin and HIAA concentrations were determined by HPLC-CEAS as described in Materials and Methods. Each data point represents the mean ± SE of 5–7 animals. \* Values are significantly different from respective controls at  $p < 0.05$ , †  $p > 0.05$  but  $< 0.10$ .

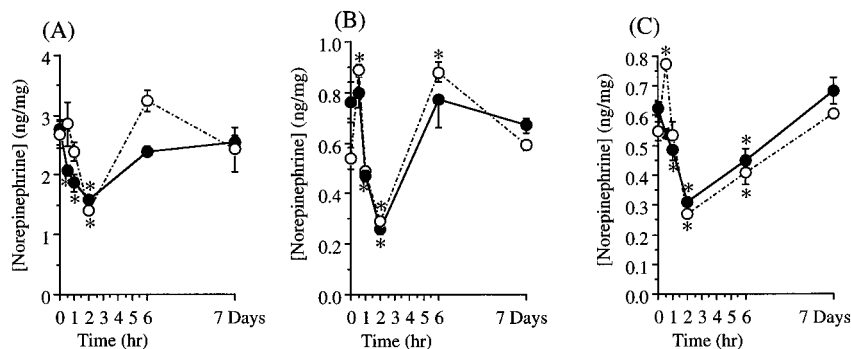
BUI ( $48.8 \pm 3.8\%$ , mean ± SD,  $n = 4$ ; Figure 7). GSH (1 mM) decreased the BUI for 5-(glutathion-S-yl)-[ $^3\text{H}$ ]-α-MeDA, in control and acivicin-treated animals, to  $4.0 \pm 0.9\%$  and  $2.5 \pm 0.8\%$ , respectively (mean ± SD,  $n = 3$

or 4), suggesting that GSH and 5-(glutathion-S-yl)-[ $^3\text{H}$ ]-α-MeDA may share the same carrier.

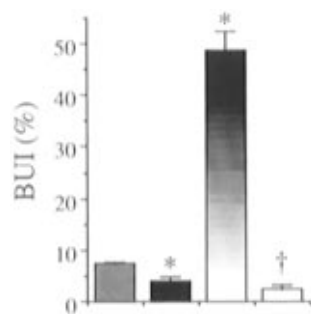
## Discussion

We have shown that a single icv administration of 5-(glutathion-S-yl)-α-MeDA (720 nmol) to male Sprague-Dawley rats causes short-term changes in neurotransmitter levels similar to those seen after subcutaneous administration of MDA (20 mg/kg, 23 μmol/rat). Moreover, the behavioral changes observed after 5-(glutathion-S-yl)-α-MeDA (Table 1) are compatible with it contributing to the behavioral and/or neurotoxic effects of MDA and MDMA. Behaviors such as forepaw treading, Straub tail, and head jerking/swaying are behaviors associated with a serotonin excess, or the "Serotonin Syndrome" (36). Consistent with the behavioral changes, 5-(glutathion-S-yl)-α-MeDA also caused an increase in the turnover of 5-HT, as evidenced by increases in 5-HT concentrations in the midbrain/diencephalon/telencephalon (Figure 4) and by increases in 5-HIAA in the midbrain/diencephalon/telencephalon (Figure 4), cortex, hippocampus, and striatum (Figure 5).

Both MDA and 5-(glutathion-S-yl)-α-MeDA caused an increased turnover of brain dopamine (Figures 1–3). An increase in dopamine synthesis has been implicated as a prerequisite for the long-term depletion of brain 5-HT following MDMA administration. In support of this view, pharmacological interventions, which inhibit either dopamine synthesis or the reuptake of synaptic dopamine, block the long-term depletion of brain 5-HT by MDMA (37). The tyrosine hydroxylase inhibitor, α-methyl-*p*-tyrosine, also significantly attenuated MDMA-mediated striatal dopamine release and blocked the long-term depletion of striatal 5-HT, but inhibition of tryptophan hydroxylase with *p*-chlorophenylalanine failed to protect against the long-term neurotoxic effects of MDMA (38). Thus, acute depletion of dopamine, but not of 5-HT, is protective against MDMA neurotoxicity and supports the hypothesis that dopamine plays a major role in the serotonergic toxicity of MDMA. However, although 5-(glutathion-S-yl)-α-MeDA reproduced some of the effects of MDA on the dopaminergic system and was capable of causing acute increases in 5-HT turnover, the current protocol did not result in long-term serotonergic toxicity. However, in contrast to MDA, 5-(glutathion-S-yl)-α-MeDA caused a rapid and significant increase in brain DOPAC (Figures 2 and 3), perhaps because 5-(glutathion-S-yl)-α-MeDA prevents vesicular dopamine uptake. Hence, not all components of the MDA-mediated



**Figure 6.** Time-dependent effects of (○) MDA (93 μmol/kg, sc) and (●) 5-(glutathion-S-yl)-α-MeDA (720 nmol, icv) on norepinephrine concentrations in hypothalamus (A), midbrain/diencephalon/telencephalon (B), and pons/medulla (C). Norepinephrine concentrations were determined by HPLC-CEAS as described in Materials and Methods. Each data point represents the mean ± SE of 5–7 animals. Data were analyzed by ANOVA followed by a *post hoc* Student Newman-Kuels test. \* Values are significantly different from respective controls at  $p < 0.05$ .



**Figure 7.** The effect of reduced glutathione (1 mM) and acivicin (18 mg/kg) on the brain uptake index (BUI) of 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA (8.5  $\mu$ M). Data represent the mean  $\pm$  SD of 3–5 experiments. (Gray bar) 5-(Glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA; (■) 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA with 1 mM glutathione; (black to white shaded bar) 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA with acivicin (18 mg/kg); (□) 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA with 1 mM glutathione and acivicin (18 mg/kg). \*Significantly different from animals injected with 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA (8.5  $\mu$ M) alone,  $p < 0.05$ . #Significantly different from animals injected with 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA (8.5  $\mu$ M) and acivicin.

effects on the brain dopaminergic system are reproduced by 5-(glutathion-S-yl)- $\alpha$ -MeDA. HVA concentrations also initially declined following MDA administration, but in contrast to DOPAC, HVA eventually increased. These differences may partially explain the inability of a single dose of 5-(glutathion-S-yl)- $\alpha$ -MeDA to produce long-term serotonergic toxicity. Alternatively, acute stimulation of dopamine turnover after MDA administration may be a necessary but insufficient event in the long-term serotonergic toxicity.

MDA is also known to produce a pressor response that can be blocked either by depleting endogenous norepinephrine stores with reserpine, or by administration of phenoxybenzamine, an  $\alpha$ -adrenergic antagonist (35), indicating that this action of MDA is related to its ability to release neuronal norepinephrine stores. Indeed, the potent adrenergic effect of MDA is long established (39). Both MDA and 5-(glutathion-S-yl)- $\alpha$ -MeDA caused depletion of brain norepinephrine concentrations (Figure 6). Thus, a single icv administration of 5-(glutathion-S-yl)- $\alpha$ -MeDA was able to mimic short-term changes in norepinephrine levels seen after peripheral administration of MDA.

The finding that  $\alpha$ -MeDA itself does not produce the same behavioral profile as 5-(glutathion-S-yl)- $\alpha$ -MeDA (Table 1) indicates that the behavioral changes are not simply a consequence of the catecholamine function. Although the cytoprotective effects of GSH are well established, and GSH and related enzymes participate in the protection of neurons from a variety of stresses, additional roles for GSH in brain function are being reported which corroborate the association between changes in GSH metabolism, and the development of certain neurodegenerative processes of the brain, including ischemia and Parkinson's disease. For example, several studies have demonstrated the existence of GSH binding sites within the mammalian CNS (40), suggesting a possible role for GSH as a neuromodulator or neurotransmitter, and in rat brain slices depolarization induces GSH secretion into the extracellular space in a  $\text{Ca}^{2+}$ -dependent manner (41). Both GSH and GSSG modulate the NMDA receptor (42), and GSH also modulates  $\mu$ -opioid, substance P/neurokinin-1, and kainic acid receptor binding sites (43). GSH and GSSG also elicit a potent antinociceptive activity (44). Consistent with a

role for GSH in receptor-mediated events, GSH produces an increase in second messenger synthesis (inositol 1,4,5-triphosphate) when added to cultured rat brain astrocytes (45). GSH may also be required to maintain the uptake of catecholamines into synaptosomes (46) and accelerates sodium channel inactivation in excised rat axonal membranes (47). Thus, GSH (and GSSG) appear to play important functional roles in the CNS. The unique structure of polyphenolic-GSH conjugates such as 5-(glutathion-S-yl)- $\alpha$ -MeDA permits toxicological and pharmacological activity as a consequence of either the polyphenolic (catecholamine) or GSH moiety. Toxicological sequelae may result from the electrophilic and redox properties of the quinone, whereas neuropharmacological changes may result from either the catecholamine or GSH moiety.

Because  $\gamma$ -GT is present in high concentrations in the brain, particularly on endothelial cells that form the blood-brain barrier (48), and because there appears to be a transporter capable of transferring GSH conjugates from the circulation into the brain (49), systemic formation of 5-(glutathion-S-yl)- $\alpha$ -MeDA followed by uptake into, and metabolism by, the brain may provide a mechanism to explain the role of metabolism in MDA- and MDMA-mediated neurotoxicity. The BUI for 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA (8.5  $\mu$ M) was 7.35  $\pm$  0.50% (Figure 7). This value compares with the BUI for GSH (10  $\mu$ M) of 8.3  $\pm$  3.2% reported by Cornford *et al.* (50). Subsequently, Kannan *et al.* (51) reported that the BUI for tracer GSH (in the presence of DTT) was highly dependent on age. We used animals with a mean weight of 273 g, which corresponds to ~65–75 days of age. For animals of equivalent age, Kannan *et al.* (51) reported a BUI for GSH of 10.9  $\pm$  0.2% to 12.2  $\pm$  1.6%. Our value of 7.35  $\pm$  0.50% for 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA is therefore a little lower than the BUI for GSH reported by Kannan *et al.* (51), but identical to that reported by Cornford *et al.* (50). GSH (1 mM) decreased the BUI for 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA to 4.03  $\pm$  0.88% (Figure 7), a 45% decrease, suggesting that GSH and 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA may share the same carrier. Pretreatment of animals with acivicin (AT-125, 18 mg/kg) 20 min prior to injection of 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA caused a substantial increase in the BUI (48.8  $\pm$  3.8%, Figure 7). Inhibition of  $\gamma$ -GT at the blood-brain barrier may decrease the metabolism of 5-(glutathion-S-yl)-[<sup>3</sup>H]- $\alpha$ -MeDA and increase the pool available for transport by the putative GSH carrier. This is an important finding because we have previously reported that structurally related polyphenolic-GSH conjugates decrease the activity of  $\gamma$ -GT (52). Thus, prolonged exposure of the blood-brain barrier to 5-(glutathion-S-yl)- $\alpha$ -MeDA and other thioether metabolites may result in decreased  $\gamma$ -GT activity with a subsequent increase in the uptake of the conjugates into brain.

About 1.6% of a dose of MDA (23  $\mu$ mol, sc) was excreted in bile as 5-(glutathion-S-yl)- $\alpha$ -MeDA, within 5 h.<sup>2</sup> This translates into ~50% of the dose that caused both neurobehavioral (Table 1) and neurochemical changes (we have not yet determined whether lower doses would cause similar effects). Because of the reactivity of polyphenolic-GSH conjugates, quantitation of their biliary and urinary excretion represents a minimum estimate of *in vivo* formation. In support of this view, following administration of 2-hydroxy-1-(glutathion-S-yl)-

<sup>2</sup> R. T. Miller, S. S. Lau, and T. J. Monks, unpublished data.

17 $\beta$ -estradiol, only 15% of the dose was recovered in urine, and 5% in feces, several days after administration (53). In addition, dependent upon the dose and substrate, 26–96% of an infused dose of polyphenolic–GSH conjugates is retained in the animal in the *in situ* perfused rat kidney model (54, 55). However, assuming a minimum 1.6% conversion of MDA to 5-(glutathion-S-yl)- $\alpha$ -MeDA<sup>2</sup> and a minimum brain uptake index of ~7.5% (Figure 7), a minimum of 28 nmol of 5-(glutathion-S-yl)- $\alpha$ -MeDA may gain access to the brain. Because only 7 nmol of 5-(N-acetyl-L-cystein-S-yl)- $\alpha$ -MeDA (icv) produces behaviors identical to those produced by MDA,<sup>2</sup> the metabolism data fall within the range necessary to support a potential role for these metabolites in MDA-mediated neurotoxicity. If one also considers (i) the multiple dosing regimens typical in animal models of MDA and MDMA neurotoxicity, (ii) metabolism of MDA to polyphenolic–GSH conjugates > 1.6%, (iii) and the fact that the potential inhibition of  $\gamma$ -GT by 5-(glutathion-S-yl)- $\alpha$ -MeDA (and its metabolites) will increase the BUI for 5-(glutathion-S-yl)- $\alpha$ -MeDA, then some combination of these factors will increase the likelihood for these metabolites to cause adverse effects in the brain following peripheral MDA administration.

We have also recently shown that 5-(glutathion-S-yl)- $\alpha$ -MeDA is metabolized by brain to 5-(cystein-S-yl)- $\alpha$ -MeDA and 5-(N-acetyl-L-cystein-S-yl)- $\alpha$ -MeDA, demonstrating that the brain possesses a functional mercapturic acid pathway (29). Because metabolism of polyphenolic–GSH conjugates through the mercapturic acid pathway can influence their redox properties (reactivity) (56), our data may provide a biochemical basis for the heterogeneity in response of the brain to certain neurotoxicants and, also, provide a basis for further investigation of the role of the mercapturic acid pathway in MDA- and MDMA-mediated neurotoxicity.

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