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Metabolism of 5-(Glutathion-*S*-yl)-α-methyldopamine Following Intracerebroventricular Administration to Male Sprague-Dawley Rats

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5-(Glutathion-*S*-yl)-α-methyldopamine [5-(GSyl)-α-MeDA] is a putative metabolite of the serotonergic neurotoxicants 3,4-(±)-(methylenedioxy)amphetamine and 3,4-(±)-(methylenedioxy)methamphetamine. Glutathione (GSH) conjugates of several polyphenols are biologically (re)active. Therefore, as part of our studies on the role of 5-(GSyl)-α-MeDA in MDA-mediated neurotoxicity, we determined the regional brain metabolism of 5-(GSyl)-α-MeDA (720 nmol) following intracerebroventricular administration to male Sprague-Dawley rats. 5-(GSyl)-α-MeDA was rapidly cleared from all brain regions examined, and regional differences in the distribution of γ-glutamyl transpeptidase (γ-GT) correlated with the formation of 5-(cystein-*S*-yl)-α-methyldopamine (5-[CYS]-α-MeDA). We also observed the formation of 5-(*N*-acetyl-L-cystein-*S*-yl)-α-MeDA (5-[NAC]-α-MeDA) in all brain regions, indicating that the brain has the ability to synthesize mercapturic acids. Peak concentrations of 5-(NAC)-α-MeDA were found in the order: hypothalamus > midbrain/diencephalon/telencephalon > pons/medulla > hippocampus > cortex > striatum. In contrast to 5-(GSyl)-α-MeDA and 5-(CYS)-α-MeDA, 5-(NAC)-α-MeDA was eliminated relatively slowly from the brain. Differences were also found in cysteine conjugate *N*-acetyltransferase activity in microsomes prepared from the various brain regions, but little difference was observed in brain cytosolic *N*-acetyl-L-cysteine conjugate *N*-deacetylase activity. We propose that some of the acute effects of 3,4-(±)-(methylenedioxy)-amphetamine and 3,4-(±)-(methylenedioxy)methamphetamine may be a consequence of the initial high concentrations of 5-(CYS)-α-MeDA, followed by the accumulation and persistence of 5-(NAC)-α-MeDA, which contributes to the long-term neurotoxicity. Because the mercapturic acid pathway can regulate the reactivity of quinones, our data may provide a biochemical basis for the heterogeneity in response of the brain to certain neurotoxicants.

Introduction

3,4-(±)-(Methylenedioxy)amphetamine (MDA)[1] and 3,4-(±)-(methylenedioxy)methamphetamine (MDMA) are serotonergic neurotoxicants in several animal species, including nonhuman primates (*1*–*3*). In recent years, their clandestine manufacture and appearance on the street have made them popular drugs of abuse (*4*–*6*). When injected directly into brain, MDA and MDMA do not produce neurotoxicity, suggesting that systemic metabolism plays an important role in the development of toxicity (*7*). However, intracerebral administration of α-methyldopamine (α-MeDA) and 3-*O*-methyl-α-methyldopamine, major metabolites of MDA, fails to reproduce the neurotoxicity of the parent compound (*8*). In addition, although the 6-hydroxydopamine analogs, 2-(methylamino)-1-(2,4,5-trihydroxyphenyl)propane (2,4,5-trihydroxymethamphetamine) and 2-amino-1-(2,4,5-trihydroxyphenyl)propane (2,4,5-trihydroxyamphetamine), putative metabolites of MDMA and MDA, respectively, caused depletion of both dopamine and serotonin following intracerebroventricular (icv) and intrastriatal administration to rats (*9*, *10*), mechanisms for their uptake into brain following systemic formation remain to be elucidated. Thus the nature of the metabolite(s) responsible for MDA- and MDMA-mediated neurotoxicity remains unclear.

Both MDA and MDMA are metabolized in vivo to α-MeDA, a reactive species (*11*, *12*) that has been shown to undergo oxidation and conjugation with glutathione (GSH) (*13*, *14*). Conjugation of electrophiles with GSH usually results in detoxication and their subsequent elimination as mercapturic acids (*15*). However, several examples exist where conjugation of GSH with electrophiles results in preservation or enhancement of biological (re)activity (*16*). For example, quinone thioethers retain the ability to redox cycle and produce reactive oxygen species, and to arylate tissue macromolecules (*17*, *18*). Quinone thioethers also inhibit enzymes which utilize GSH as a cosubstrate (*19*). In particular, the 5-(*S*-glutathionyl) conjugates of dopamine and α-methyldopa inhibit human GSH *S*-transferases in a reversible (GST M1a-1a, M1b-1b, A1-1, A2-2, and P1-1) and irreversible (GST P1-1) manner (*20*).

Because (i) neither MDA nor MDMA produce neurotoxicity when injected directly into brain (*7*), (ii) icv administration of some major metabolites of MDA and MDMA fails to reproduce their neurotoxicity (*8*), (iii) α-MeDA is a metabolite of both MDA and MDMA (*11*, *12*), (iv) α-MeDA is readily oxidized to the corresponding

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[1] Abbreviations: 3,4-(±)-(methylenedioxy)amphetamine [MDA]; 3,4-(±)-(methylenedioxy)methamphetamine [MDMA]; glutathione [GSH]; γ-glutamyl transpeptidase [γ-GT]; α-methyldopamine [α-MeDA]; 5-(glutathion-*S*-yl)-α-methyldopamine [5-(GSyl)-α-MeDA]; 5-(cystein-*S*-yl)-α-methyldopamine [5-(CYS)-α-MeDA]; 5-(*N*-acetyl-L-cystein-*S*-yl)-α-methyldopamine [5-(NAC)-α-MeDA]; HPLC coulometric electrode array system [HPLC-CEAS]; intracerebroventricular [icv].

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quinone which can undergo conjugation with GSH (*14*), and (v) quinone thioethers exhibit a variety of toxicological activities (*16*), we have initiated studies on the potential role of thioether metabolites of α-MeDA in the neurotoxicity of MDA and MDMA. As part of these studies, we have investigated the metabolism of 5-(glutathion-*S*-yl)-α-MeDA [5-(GSyl)-α-MeDA] following icv administration to male Sprague-Dawley rats. We report that 5-(GSyl)-α-MeDA is rapidly cleared from brain and that regional differences in 5-(cystein-*S*-yl)-α-MeDA (5-[CYS]-α-MeDA) formation correlate with differences in γ-glutamyl transpeptidase (γ-GT) activity. We have also identified 5-(*N*-acetyl-L-cystein-*S*-yl)-α-MeDA [5-(NAC)-α-MeDA] as a metabolite of 5-(GSyl)-α-MeDA, demonstrating that the brain has the ability to synthesize mercapturic acids. The results are discussed with respect to the potential contribution of these metabolites to the neurotoxicity of MDA and MDMA.

Materials and Methods

Chemicals. Reduced glutathione, mushroom tyrosinase (5600 units/mg), acetyl coenzyme-A, and ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO). 2-Bromohydroquinone was a product of ICN (Cleveland, OH), and 2-bromo-6-(cystein-*S*-yl)- and 2-bromo-6-(*N*-acetyl-L-cystein-*S*-yl)hydroquinone were prepared according to previously established methodology (*21*). Cysteine and *N*-acetyl-L-cysteine were obtained from Aldrich Chemical Co. (Milwaukee, WI). α-MeDA was a generous gift from Dr. Anthony Lu, Merck Sharp and Dohme Laboratories (Rahway, NJ). 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.

Synthesis and Purification of 5-(Glutathion-S-yl)-α-MeDA. 5-(GSyl)-α-MeDA was prepared according to previously published methods (*13*, *22*), with modifications. Briefly, a mixture of α-MeDA (2 mM), GSH (10 mM), and mushroom tyrosinase (100 units/mL) was stirred at 25 °C for 30 min in 100 mL of sodium phosphate buffer (50 mM, pH 7.4). The solution was poured directly onto Sep-Pak C18 cartridges (Waters Associates, Milford, MA), total volume 10 mL/cartridge, which had been pretreated sequentially with 5 mL of methanol, 5 mL of water, and 3 mL of sodium phosphate buffer (50 mM, pH 7.4). The cartridges were then washed with 1.5 mL of water, and 5-(GSyl)-α-MeDA was eluted with 3 mL of formic acid/water/methanol (1:49:50). The eluates were combined, concentrated by rotary evaporation, frozen over dry ice/acetone, and lyophilized to dryness. The resulting product was further purified by HPLC (Shimadzu, LC-6A) by dissolving in 10 mL of 1% formic acid (ca. 5 mg/mL) and injecting 1 mL aliquots onto a Beckman Ultrasphere ODS-5 reverse-phase semipreparative column (25 × 1 cm i.d.; 5 μm particle size). The product was eluted using formic acid/water/methanol (1:94:5) at a flow rate of 3 mL/min. The fractions containing the one major UV absorbing product (λ = 280 nm) were combined, concentrated by rotary evaporation, frozen over dry ice/acetone, and lyophilized to dryness. The resulting powder, when analyzed with an HPLC coulometric electrode array system (HPLC-CEAS) or HPLC with UV detection, gave rise to a single product. The UV spectrum of the purified compound was recorded on a Shimadzu UV 160U spectrophotometer and the structure confirmed by ^{1}H-NMR. 5-(GSyl)-α-MeDA was obtained as a pale tan powder. In 1% formic acid, 5-(GSyl)-α-MeDA exhibited a UV spectrum with λ_{max}, nm (log ϵ_{max}, M^{-1} cm^{-1}), at 292 (3.42), 256 (3.57), and 236 (3.66). ^{1}H-NMR (D_2O) δ 6.77 (d, J = 1.63 Hz, 1H, H_6), 6.68 (d, J = 1.51 Hz, 1H, H_2), 4.30 (m, 1H, Cys-α), 3.62 (t, 1H, Glu-α), 3.48 (d, 2H, Gly-α), 3.44 (m, 1H, CH), 3.26 (dd, 1H, Cys-β), 3.10 (m, 1H, Cys-β), 2.67 (m, 2H, CH_2), 2.35 (t, 2H, Glu-γ), 1.98 (dd, 2H, Glu-β), 1.17 (d, 3H, CH_3).

Synthesis and Purification of 5-(*N*-Acetyl-L-cystein-*S*-yl)-α-MeDA. α-MeDA (2 mM), *N*-acetyl-L-cysteine (10 mM), and mushroom tyrosinase (132 units/mL), in a total volume of 50 mL of sodium phosphate buffer (50 mM, pH 7.4), were vigorously stirred for 3 min at room temperature. The reaction was quenched by addition of 2 mL of 88% formic acid, frozen over dry ice/acetone, and lyophilized to dryness. The resulting crude product was purified by HPLC (Shimadzu, LC-6A) by dissolving in 10 mL of 1% formic acid (ca. 3 mg/mL) and injecting 1 mL aliquots onto a Beckman Ultrasphere ODS-5 reverse-phase semipreparative column. The product was eluted using the following MeOH/water gradient: 0−20 min linear gradient from 10% to 20% MeOH; 20−25 min linear gradient from 20% to 30% MeOH, flow rate of 3 mL/min. Fractions containing the one major UV absorbing product (λ = 280 nm) were combined, concentrated by rotary evaporation, frozen over dry ice/acetone, and lyophilized to dryness. The resulting powder, when analyzed by HPLC-CEAS or HPLC with UV detection, gave rise to a single product. 5-(NAC)-α-MeDA was obtained as a white, fluffy powder. In 1% formic acid, 5-(NAC)-α-MeDA exhibited a UV spectrum with λ_{max}, nm (log ϵ_{max}, M^{-1} cm^{-1}), at 292 (3.40), 256 (3.54), and 235 (3.67). ^{1}H-NMR (D_2O) δ 6.80 (d, J = 1.98 Hz, 1H, H_6), 6.67 (d, J = 1.96 Hz, 1H, H_2), 4.11 (m, 1H, Cys-α), 3.45 (m, 1H, CH), 3.28, 2.97 (m, 2H Cys-β), 2.68 (m, 2H, CH_2), 1.76 (d, 3H, acetyl CH_3), 1.18 (dd, 3H, CH_3).

Intracerebroventricular Administration of 5-(GSyl)-α-MeDA. 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 their heads shaved and placed in a stereotaxic apparatus. A midsaggital 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 intra-aural 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 μL artificial cerebrospinal fluid (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 1.2 mM, and $MgSO_4$ 1.2 mM) as the vehicle. 5-(GSyl)-α-MeDA (720 nmol) was infused into the left lateral ventricle of the awake animals at a rate of 2 μL every 30 s (total volume 10 μL) 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 μL artificial cerebrospinal fluid (icv). Following icv administration of 5-(GSyl)-α-MeDA, animals were euthanized by decapitation at 0.25, 0.5, 1, 2, 6, and 168 h and their brains quickly removed and placed onto a chilled plate. The occipital cortex, hippocampus, striatum, hypothalamus, and areas of the brainstem corresponding to the midbrain/diencephalon/telencephalon and pons/medulla 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 rich in serotonergic and/or dopaminergic nerve terminal fields and which correspond to 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 °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 μM EDTA and 263 μM $Na_2S_2O_5$ for 30 s with a cell disrupter. The sonicated tissue was then centrifuged at 13500*g* for 10 min in a table-top microcentrifuge (Eppendorf; Model 5415c). Supernatants were then filtered through 0.22 μm nylon filters (Acrodisc-13, Gelman Sciences), and 20 μL of the filtrate was analyzed by HPLC-CEAS.

HPLC-CEAS Analysis of 5-(GSyl)-α-MeDA and Metabolites. Separation and quantitation of 5-(GSyl)-α-MeDA, 5-(CYS)-α-MeDA, and 5-(NAC)-α-MeDA was accomplished by HPLC-CEAS. The mobile phase consisted of 4 mM citrate, 8 mM ammonium acetate, 54 μM EDTA, and 5% methanol (pH 3.0). Separation was accomplished by use of an ESA HR-80 column (80 × 4.6 mm i.d.; 3 μm particle size). The flow rate was held constant at 1 mL/min. Retention times (in parentheses) for the compounds were as follows: 5-(CYS)-α-MeDA (5.75 min), 5-(GSyl)-α-MeDA (9.5 min), and 5-(NAC)-α-MeDA (18 min). Quantitation of 5-(GSyl)-α-MeDA and 5-(NAC)-α-MeDA was accomplished by comparison of peak areas with standard curves generated from authentic standards during each series of HPLC analyses. Pure samples of 5-(CYS)-α-MeDA could not be obtained for structural analysis due to breakdown of the compound (presumably a consequence of autoxidation) during preparative workup. Therefore, quantitation of 5-(CYS)-α-MeDA is expressed as 5-(GSyl)-α-MeDA equivalents based upon the following rationale: (i) the retention time of 5-(CYS)-α-MeDA was verified by coelution of sample with an aliquot of a freshly prepared synthetic reaction mixture of cysteine, tyrosinase, and α-MeDA; (ii) the electrochemical properties of 5-(GSyl)-α-MeDA (channel 1 [0 mV]:channel 2 [+50 mV] = 0.22), synthetic 5-(CYS)-α-MeDA (channel 1 [0 mV]:channel 2 [+50 mV] = 0.21), and the sample peak eluting with the same retention time as synthetic 5-(CYS)-α-MeDA (channel 1 [0 mV]:channel 2 [+50 mV] = 0.21) were identical, as determined by the behavior of the compounds across the coulometric array, confirming that peak areas per nmol equivalents of each compound are similar at the potentials employed in the HPLC-CEAS assay.

γ-Glutamyl Transpeptidase. Rat brains were dissected and regions pooled and homogenized in 5 volumes of Tris-KCl buffer (pH 7.4; 0.15 M KCl; 20 mM Tris) at 4 °C. Aliquots (20 μL) of the homogenates were used for the γ-GT assay. γ-GT activity in regional brain homogenates was assayed by determining the rate of *p*-nitroaniline formation from γ-glutamyl-*p*-nitroaniline in 0.1 M Tris (pH 9.0) as previously described (*23*). Protein concentration was determined by the method of Lowry (*24*) using bovine serum albumin as a standard.

Preparation of Microsomal and Cytosolic Fractions. Animals were euthanized by decapitation and their brains quickly removed onto a chilled plate. The brains were rapidly dissected into whole cortex, hippocampus, striatum, hypothalamus, midbrain/diencephalon/telencephalon, and pons/medulla. Brain regions were homogenized in Tris-KCl buffer (1:5 w/v) at 4 °C. The homogenates were centrifuged at 10000*g* for 20 min, and the 10000*g* supernatants were further centrifuged at 100000*g* for 1 h to obtain the microsomal and cytosolic fractions.

Determination of Brain *N*-Acetyl-L-cysteine Conjugate *N*-Deacetylase. *N*-Deacetylation was determined according to previously described methodology (*21*, *25*). Briefly, incubation mixtures contained 3.33 mg/mL brain cytosolic protein, 1 mM ascorbic acid, and 100 μM 2-bromo-6-(*N*-acetyl-L-cystein-*S*-yl)-hydroquinone, in Tris-KCl (pH 7.4). The mixtures were preincubated at 37 °C for 2 min and the reactions initiated by addition of substrate and incubated for a further 10 min. Control incubations contained boiled tissue. Incubations were terminated by the addition of 0.1 mL of 10% perchloric acid to 1.5 mL aliquots of the incubation mixture. The mixtures were vortex mixed, placed on ice, and centrifuged at 13500*g* for 10 min to pellet denatured protein. The acidic supernatants were stored at −70 °C until analysis (within 1 week). Aliquots (20 μL) of samples were injected onto a Partisil 5 ODS-3 reverse-phase column (25 cm × 4.6 mm; 5 μm particle size; Whatman) and eluted with a mobile phase of methanol (0−50% linear gradient over 25 min): 4 mM citrate, 8 mM ammonium acetate, and 134 μM EDTA (pH 4.0). The flow rate was 1 mL/min. The eluate was monitored at +50 mV. Under these conditions, the retention times (in parentheses) were as follows: 2-bromo-6-(cystein-*S*-yl)hydroquinone (13.5 min) and 2-bromo-6-(*N*-acetyl-L-cystein-*S*-yl)hydroquinone (17.25 min). For quantitation, peak areas were compared with a standard curve generated from authentic standards during each series of HPLC analyses.

Determination of Brain Microsomal Cysteine Conjugate *N*-Acetyltransferase. *N*-Acetylation was determined with freshly prepared rat brain microsomes with 100 μM substrate, 200 μM CoASAc, 1 mM ascorbic acid, and 1 mg/mL microsomal protein, in a final volume of 0.5 mL of Tris-KCl (pH 7.4) at 37 °C. The mixtures were preincubated for 2 min at 37 °C and the reactions initiated by addition of 2-bromo-6-(cystein-*S*-yl)hydroquinone. The mixtures were then incubated for 5 min at 37 °C. Control incubations contained boiled tissue. The reactions were terminated by addition of 1.5 mL of 1.33 M acetic acid. The mixtures were vortex mixed, placed on ice, and centrifuged to pellet denatured protein. The acidic supernatants were analyzed by HPLC-CEAS as described above.

Results

Animal behavior was monitored following icv administration of 5-(GSyl)-α-MeDA (720 nmol; doses >720 nmol were lethal). 5-(GSyl)-α-MeDA-treated animals became hyperactive and aggressive and displayed forepaw treading and Straub tails and head jerking/swaying[2] behaviors associated with a serotonin excess or the "Serotonin Syndrome" (*26*, *27*). Such behaviors are consistent with a role for 5-(GSyl)-α-MeDA in some of the behavioral alterations seen after administration of serotonin releasers, such as MDA and MDMA.

Metabolism of 5-(GSyl)-α-MeDA. Following icv administration, 5-(GSyl)-α-MeDA (720 nmol) was rapidly cleared from all regions of the brain examined (Figures 1−4). Because 5-(GSyl)-α-MeDA is delivered directly into the left lateral ventricle, it will diffuse rapidly into all areas of the brain following icv injection. The CSF is found within all four ventricles, and small solutes diffuse freely between the extracellular fluid and the CSF, facilitating the movement of metabolites from deep within the hemispheres to cortical subarachnoid spaces and the ventricular system. Concomitant with the rapid disappearance of 5-(GSyl)-α-MeDA from the brain, 5-(CYS)-α-MeDA accumulated rapidly (Figures 1−4), reaching higher concentrations at sites ipsilateral to the site of injection (Figures 1−3). Maximum concentrations of 5-(CYS)-α-MeDA were reached between 30 and 60 min after 5-(GSyl)-α-MeDA administration and were highest in the hippocampus (351 ± 67 pmol/mg), followed by the hypothalamus (284 ± 25 pmol/mg), striatum (156 ± 16 pmol/mg), pons/medulla (118 ± 9 pmol/mg), midbrain/diencephalon/telencephalon (87 ± 8 pmol/mg), and cortex (80 ± 9 pmol/mg).

5-(CYS)-α-MeDA was also rapidly eliminated from the brain, with the concomitant formation of 5-(NAC)-α-MeDA, which reached maximal concentrations 120 min after 5-(GSyl)-α-MeDA administration. The highest concentrations of 5-(NAC)-α-MeDA were found in the hypothalamus (154 ± 9 pmol/mg) followed by the hippocampus (119 ± 33 pmol/mg), midbrain/diencephalon/telencephalon (104 ± 12 pmol/mg), pons/medulla (67 ± 6 pmol/mg), cortex (33 ± 13 pmol/mg), and striatum (30 ± 6 pmol/mg). However, in contrast to 5-(GSyl)-α-MeDA

[2] R. T. Miller, S. S. Lau, and T. J. Monks. Manuscript submitted.

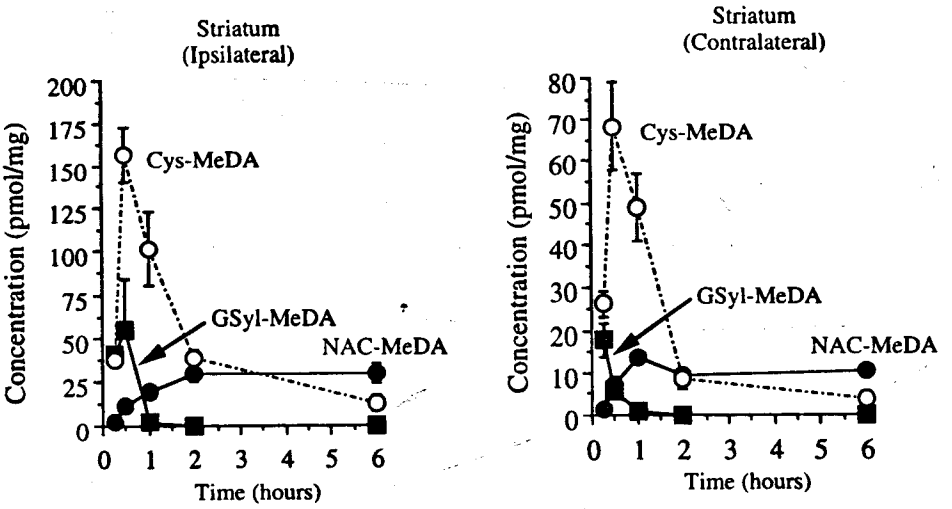

Figure 1. Metabolism of 5-(glutathion-*S*-yl)-α-methyldopamine (720 nmol) in rat striatum ipsilateral and contralateral to the site of intracerebroventricular injection. Each data point represents the mean ± SE of 5–7 animals.

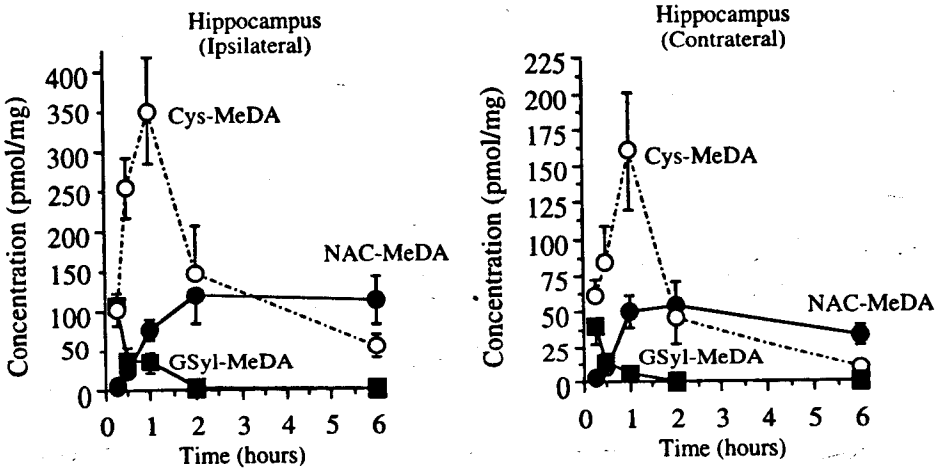

Figure 2. Metabolism of 5-(glutathion-*S*-yl)-α-methyldopamine (720 nmol) in rat hippocampus ipsilateral and contralateral to the site of intracerebroventricular injection. Each data point represents the mean ± SE of 5–7 animals.

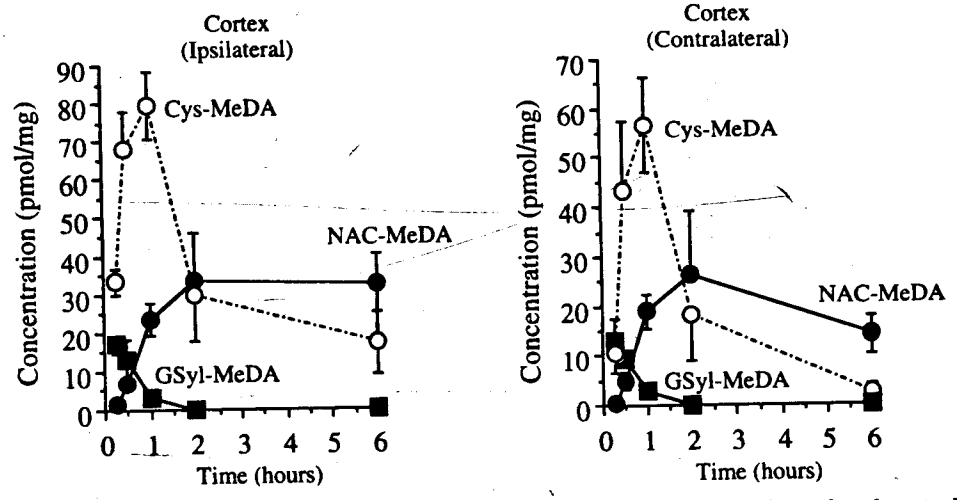

Figure 3. Metabolism of 5-(glutathion-*S*-yl)-α-methyldopamine (720 nmol) in rat cortex ipsilateral and contralateral to the site of intracerebroventricular injection. Each data point represents the mean ± SE of 5–7 animals.

and 5-(CYS)-α-MeDA, 5-(NAC)-α-MeDA was eliminated relatively slowly from the brain (Figure 5); between 2 and 6 h following administration of 5-(GSyl)-α-MeDA, 5-(NAC)-α-MeDA concentrations remained virtually unchanged in the pons/medulla, cortex, striatum, and hippocampus. Only in the midbrain/diencephalon/telencephalon and hypothalamus (Figure 4) did 5-(NAC)-α-MeDA concentrations decline significantly between 2 and 6 h (31% and 63%, respectively).

The first step in the metabolism of GSH and its corresponding S-conjugates requires the activity of γ-GT, and therefore concentrations of 5-(CYS)-α-MeDA and 5-(NAC)-α-MeDA in each brain region should reflect regional differences in the distribution of this enzyme.

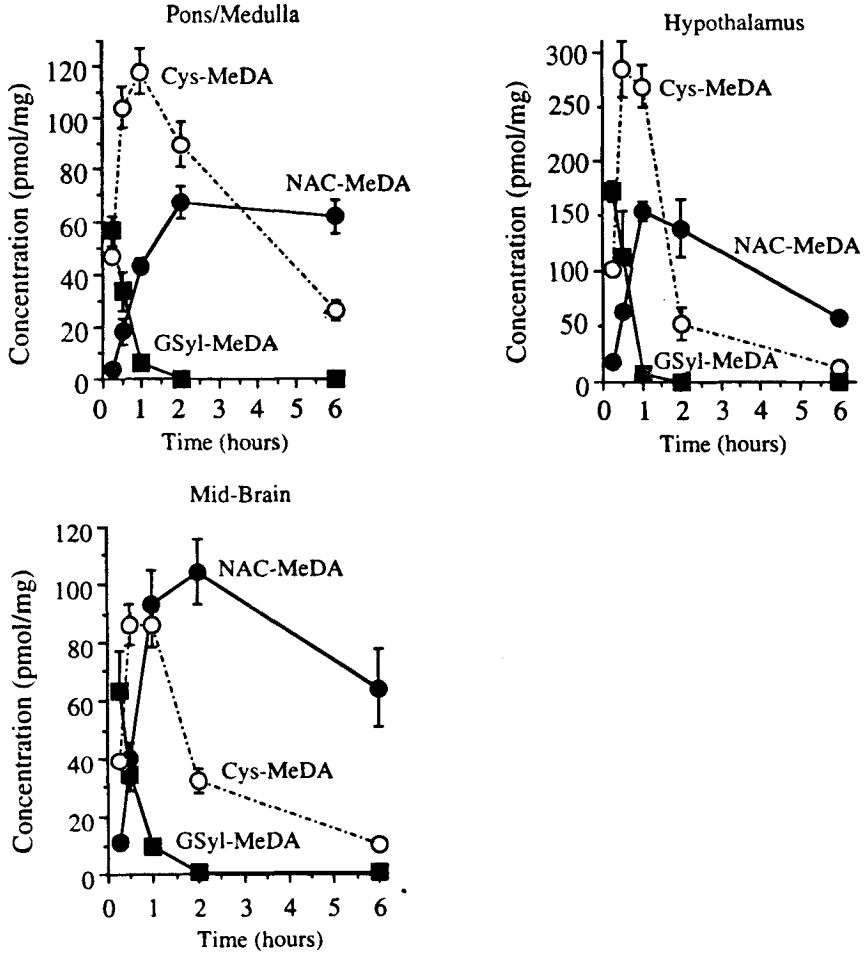

Figure 4. Metabolism of 5-(glutathion-*S*-yl)-α-methyldopamine (720 nmol) in the pons/medulla, hypothalamus, and midbrain/diencephalon/telencephalon following intracerebroventricular administration to rats. Each data point represents the mean ± SE of 5−7 animals.

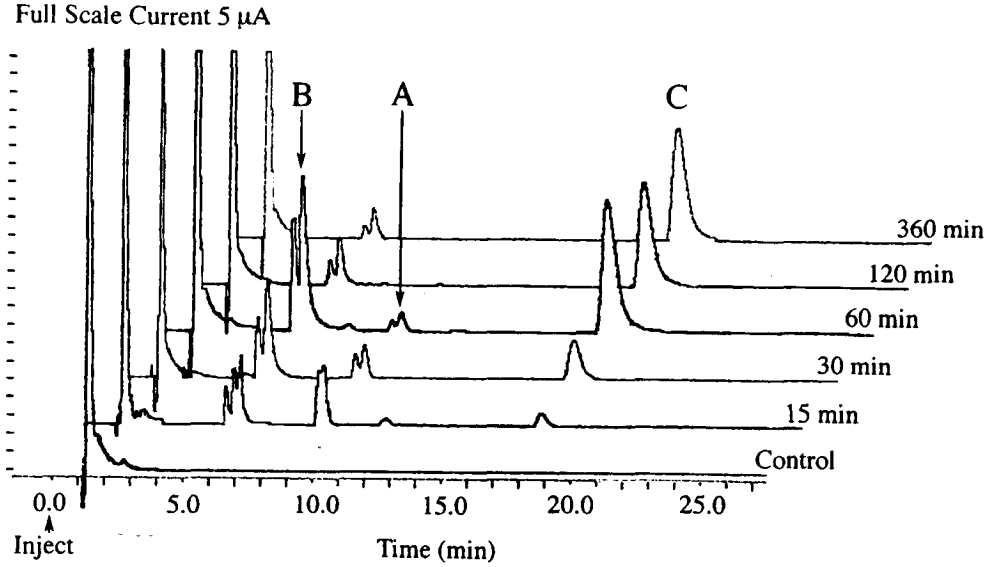

Figure 5. HPLC-CEAS analysis illustrating the rapid metabolism and elimination of 5-(glutathion-*S*-yl)-α-methyldopamine (A) in the midbrain/diencephalon/telencephalon with the concomitant formation and elimination of 5-(cystein-*S*-yl)-α-methyldopamine (B). 5-(*N*-Acetylcystein-*S*-yl)-α-methyldopamine (C) is the final redox active metabolite formed in brain, and it is eliminated relatively slowly. Assignment of the identity of each peak was based upon both the retention time and electrode pair accuracy. HPLC conditions are as described in Materials and Methods, and the chromatogram represents samples obtained from animals at the different time points indicated, monitored at +50 mV.

Consistent with this view, regional differences in brain γ-GT activity (Table 1) correlated with the total 5-(CYS)-α-MeDA$_{AUC}$ and 5-(NAC)-α-MeDA$_{AUC}$ (Figure 6; $r = 0.92$; $p < 0.05$). Although the combined concentrations of 5-(CYS)-α-MeDA and 5-(NAC)-α-MeDA in the brain will be dependant upon the activity of γ-GT, their relative concentrations will depend upon the relative activities of brain cysteine conjugate *N*-acetyltransferase and *N*-

Table 1. Regional γ-Glutamyl Transpeptidase Activity in the Male Sprague-Dawley Rat[a]

region	nmol/(min·mg)
cortex	3.3 ± 0.03
striatum	4.2 ± 0.09
midbrain/diencephalon/telencephalon	4.2 ± 0.04
hypothalamus	5.0 ± 0.09
pons/medulla	5.4 ± 0.15
hippocampus	6.7 ± 0.14

[a] Each value represents the mean ± SE; $N = 3$.

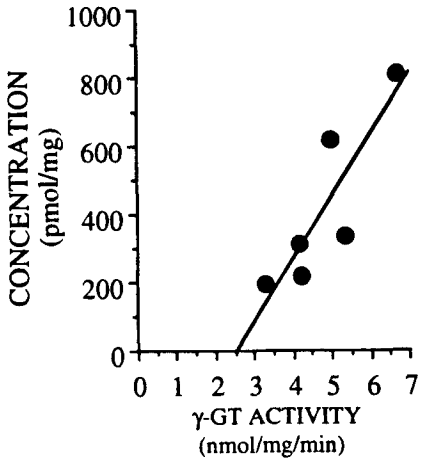

Figure 6. Correlation between regional differences in brain γ-glutamyl transpeptidase activity and the formation of 5-(cystein-*S*-yl)-α-methyldopamine and 5-(*N*-acetylcystein-*S*-yl)-α-methyldopamine.

Table 2. Regional CNS *N*-Acetylation/*N*-Deacetylation in the Male Sprague-Dawley Rat[a]

region	*N*-acetylation	*N*-deacetylation	ratio
striatum	76 ± 2.7	43 ± 0.5	1.8
cortex	106 ± 3.3	39 ± 2.3	2.7
hippocampus	127 ± 5.9	33 ± 0.8	3.9
hypothalamus	166 ± 3.6	44 ± 0.9	3.8
pons/medulla	190 ± 5.3	35 ± 1.3	5.4
midbrain/diencephalon/telencephalon	409 ± 22.2	33 ± 1.3	12.3

[a] Each value, in pmol/mg/min, represents the mean ± SE; $N = 3$.

acetyl-L-cysteine conjugate deacetylase. Little difference was found in the deacetylase activity between regions (Table 2), ranging from a high of 44 ± 1 pmol/(mg·min) (hypothalamus; mean ± SE; $n = 3$) to a low of 33 ± 1 pmol/(mg·min) (hippocampus and midbrain/diencephalon/telencephalon; mean ± SE, $n = 3$). In contrast, cysteine conjugate *N*-acetyltransferase activity varied from a high in the midbrain/diencephalon/telencephalon of 409 ± 22 pmol/(mg·min) to a low of 76 ± 3 pmol/(mg·min) in the striatum.

Discussion

In the present study we have shown that following icv administration of 5-(GSyl)-α-MeDA (720 nmol) to rats it is rapidly cleared from all areas of the brain examined, with the concomitant formation of 5-(CYS)-α-MeDA (Figures 1–4), peak concentrations of which correlate with regional differences in brain γ-GT activity. Although we cannot exclude the possibility that some fraction of the 5-(CYS)-α-MeDA measured in brain arises *via* peripheral metabolism of 5-(GSyl)-α-MeDA, the rapid appearance of 5-(CYS)-α-MeDA (peak concentrations are reached by 30 min), and the direct relationship between concentrations of 5-(CYS)-α-MeDA in different brain regions and the regional heterogeneity of γ-GT, suggests that the major fraction of this metabolite is generated within the brain. 5-(CYS)-α-MeDA is also rapidly eliminated from the brain, with the concomitant formation of 5-(NAC)-α-MeDA. However, by comparison to 5-(GSyl)-α-MeDA and 5-(CYS)-α-MeDA, 5-(NAC)-α-MeDA is eliminated relatively slowly from the brain. Since polyphenolic thioethers maintain the ability to redox cycle (*15–17*) and because all the thiol conjugates of α-MeDA remain susceptible to oxidation, the presence and persistence of these metabolites in brain tissue may contribute to the neurotoxicity of MDA and MDMA. Inasmuch as metabolism of MDA and MDMA appears necessary for the development of neurotoxicity, their acute effects may be a consequence of the initial high concentrations of 5-(CYS)-α-MeDA, followed by the accumulation and persistence of 5-(NAC)-α-MeDA, which then contributes to the long-term neurotoxicity. The toxicity of 5-(CYS)-α-MeDA and 5-(NAC)-α-MeDA may also be regulated by intramolecular cyclization reactions that occur subsequent to oxidation. Cyclization of 5-(CYS)-α-MeDA can occur in one of two ways. Following oxidation, the side chain (alanine-derived) amino group can cyclize to give the 5,6-dihydroxyindole, in a reaction analogous to the Raper-Mason pathway of eumelanin biosynthesis (*28–30*). Alternatively, the cysteinyl amino group can condense with the quinone carbonyl to give a benzothiazolyl-like compound, in a reaction analogous to pheomelanin synthesis (*31*). Only the latter reaction removes the reactive quinone function, since the dihydroxyindole can undergo further oxidation. The cysteinyl amino group is blocked in 5-(NAC)-α-MeDA and, following oxidation, can no longer undergo cyclization. Therefore, in addition to any inherent differences in the redox properties of 5-(CYS)-α-MeDA and 5-(NAC)-α-MeDA, the latter is likely to mainatin redox activity because it lacks the ability to undergo intramolecular detoxication (benzothiazolyl formation). Preliminary experiments with 5-(NAC)-α-MeDA have shown that this metabolite produces neurobehavioral changes consistent with those following MDA and MDMA administration, but at a dose of only 7 nmol (icv).[3]

The potential for 5-(GSyl)-α-MeDA and its metabolites to contribute to MDA- and MDMA-mediated neurotoxicity following peripheral administration will be dependent upon their ability to cross the blood–brain and blood–CSF barriers. Brain microvascular endothelial cells maintain tight junctions (*32*) and possess a high density of mitochondria (*33*), characteristics associated with renal proximal tubular epithelial cells. The mitochondria probably supply the high energy requirements for the transport of water-soluble substances through the endothelial barrier *via* specific transporters. The directionality of ion transport across the blood–brain barrier is achieved by a polarized distribution of ion channels on the endothelial cell surfaces. Therefore, endothelial cells are polarized in a manner similar to that of other transport interfaces, such as renal epithelia, with the preferential localization of specific transport systems and receptors either on the luminal or on the antiluminal side of vessel walls (*34*). For amino acids, at least three different carrier systems have been identified within the blood–brain barrier (*35*), and a variety of neurotoxicants have been shown to be transported into brain across the

[3] R. T. Miller, S. S. Lau, and T. J. Monks. Unpublished data.

blood–brain barrier *via* these amino acid carriers. For example, β-(*N*-methylamino)-L-alanine, a purported neurotoxic constituent of the cycad plant responsible for the high incidence of amyotrophic lateral sclerosis (and related parkinsonism dementia) in the western Pacific, is transported into brain *via* the large neutral amino acid carrier (*36*). The cysteine conjugate of dichloroacetylene, a potent nephrotoxicant (*37–39*) and neurotoxicant (*40*), is also transported across the blood–brain barrier by the Na^+-independent system L-transporter for neutral amino acids, while uptake of the corresponding GSH conjugate is mediated by an as yet unknown carrier system (*41*). The saturable, carrier-mediated transport of GSH across the blood–brain barrier has also been reported (*42*). Thus, transport systems that can facilitate the uptake of GSH and cysteine conjugates of α-MeDA into the brain have been described.

The nephrotoxicity of quinone thioethers has been shown to be dependent on the high concentration and localization of γ-GT within the brush border membrane of proximal tubular epithelial cells (*43–45*). γ-GT is the only enzyme known to cleave the γ-glutamyl bond of GSH resulting in the cysteinylglycine dipeptide (*46*). The γ-glutamyl moiety of the GSH conjugate is removed (extracellularly), and the resulting cysteinylglycine conjugate is converted to the cysteine conjugate. High γ-GT activities are found in tissues where amino acid transport occurs at high rates, such as in the brush border of the kidney tubular cell (*47*), in the choroid plexus (*48*), and in brain microvessels (49). The distribution of brain microvessels that express γ-GT varies within discrete regions of the brain (*50*). We have confirmed regional differences in brain γ-GT activity (Table 1), providing a biochemical basis for regional heterogeneity in response to neurotoxicants. Moreover, systemic administration of γ-glutamyl-L-3,4-dihydroxyphenylalanine increases catecholamine (dopamine and norepinephrine) levels in mouse brain (*51*), indicating that γ-GT can facilitate the uptake of polyphenols across the blood–brain barrier.

In summary, the present studies demonstrate that 5-(GSyl)-α-MeDA is metabolized by brain to 5-(CYS)-α-MeDA, which is subsequently converted to 5-(NAC)-α-MeDA. To our knowledge, this demonstrates for the first time that brain possesses a functional mercapturic acid pathway. Regional differences in the distribution of γ-GT and of microsomal cysteine conjugate *N*-acetyltransferase were documented, but little difference existed in the regional distribution of cytosolic *N*-deacetylase. Because there are changes in redox potentials during mercapturic acid formation (*21*, *52*, *53*), this pathway may regulate the reactivity of polyphenols. Our data may therefore provide a biochemical basis for the heterogeneity in the response of the brain to certain neurotoxicants. The data 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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