Serotonergic Neurotoxic Metabolites of Ecstasy Identified in Rat Brain

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ABSTRACT

The selective serotonergic neurotoxicity of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) depends on its systemic metabolism. We have recently shown that inhibition of brain endothelial cell γ-glutamyl transpeptidase (γ-GT) potentiates the neurotoxicity of both MDMA and MDA, indicating that metabolites that are substrates for this enzyme contribute to the neurotoxicity. Consistent with this view, glutathione (GSH) and N-acetylcysteine conjugates of α-methyl dopamine (α-MeDA) are selective neurotoxicants. However, neurotoxic metabolites of MDMA or MDA have yet to be identified in brain. Using in vivo microdialysis coupled to liquid chromatography-tandem mass spectrometry and a high-performance liquid chromatography-coulometric electrode array system, we now show that GSH and N-acetylcysteine conjugates of N-methyl-α-MeDA are present in the striatum of rats administered MDMA by subcutaneous injection. Moreover, inhibition of γ-GT with acivicin increases the concentration of GSH and N-acetylcycteine conjugates of N-methyl-α-MeDA in brain dialysate, and there is a direct correlation between the concentrations of metabolites in dialysate and the extent of neurotoxicity, measured by decreases in serotonin (5-HT) and 5-hydroxyindole acetic (5-HIAA) levels. Importantly, the effects of acivicin are independent of MDMA-induced hyperthermia, since acivicin-mediated potentiation of MDMA neurotoxicity occurs in the context of acivicin-mediated decreases in body temperature. Finally, we have synthesized 5-(N-acetylcycteinst-S-y1)-N-methyl-α-MeDA and established that it is a relatively potent serotonergic neurotoxicant. Together, the data support the contention that MDMA-mediated serotonergic neurotoxicity is mediated by the systemic formation of GSH and N-acetylcysteine conjugates of N-methyl-α-MeDA (and α-MeDA). The mechanisms by which such metabolites access the brain and produce selective serotonergic neurotoxicity remain to be determined.

Although the selectivity of (+)-3,4-methylenedioxymethamphetamine (MDMA, ecstasy) and (-)-3,4-methylenedioxymethamphetamine (MDA) for the serotonergic system in rats and humans is firmly established, the mechanism(s) involved are not fully understood. In rats, MDMA is cleared mainly by hepatic metabolism by N-demethylation to form MDA. MDMA and MDA are further O-demethylated to 3,4-dihydroxymethamphetamine (N-methyl-α-methyldopamine; N-Me-α-MeDA) and 3,4-dihydroxyamphetamine (α-methylamphetamine; α-MeDA), respectively. N-Me-α-MeDA and α-MeDA are highly redox-unstable catechols and are conjugated with sulfate and glucuronic acid. Both catechols can also be rapidly oxidized to their corresponding orthoquinones and form adducts with glutathione (GSH) and other thiol-containing compounds (Lim and Foltz, 1988; Hiramatsu et al., 1990). Alternatively, N-Me-α-MeDA and α-MeDA can be O-methylated to form 4-hydroxy-3-methoxyamphetamine (3-O-Me-N-Me-α-MeDA) or 4-hydroxy-3-methoxyamphetamine (3-O-Me-α-MeDA), respectively.

There is a general consensus that MDMA and MDA neuro-
NMDA and MDMA fail to produce 5-HT specific neurotoxicity. However, several known and putative metabolites of neurotoxic doses of MDMA. Following perfusion, acute monoamine release was observed in the absence of long-term depletions in 5-HT levels. These data are consistent with the hypothesis that peripheral generation of neurotoxic metabolites contributes to MDMA-induced serotoninergic neurotoxicity. However, several known and putative metabolites of MDA and MDMA fail to produce 5-HT specific neurotoxicity after intracerebroventricular (i.c.v.) injection (McCann and Ryabinin, 1989). Perhaps the most convincing evidence that peripheral metabolism of MDMA is required for neurotoxicity was provided by the work of Esteban et al. (2001), in which MDMA was perfused into the hippocampus in amounts sufficient to give rise to the range of concentrations observed following peripheral administration of neurotoxic doses of MDA. Following perfusion, acute monoamine release was observed in the absence of long-term depletions in 5-HT levels. These data are consistent with the hypothesis that peripheral generation of neurotoxic metabolites contributes to MDMA-induced serotoninergic neurotoxicity. However, several known and putative metabolites of MDA and MDMA fail to produce 5-HT specific neurotoxicity after intracerebroventricular (i.c.v.) injection (McCann and Ricaruta, 1991; Elyan et al., 1992; Johnson et al., 1992; Zhao et al., 1992).

α-MeDA and N-Me-α-MeDA are catechols that can undergo oxidation to the corresponding ortho-quinones, which are highly electrophilic, as evidenced by their ability to react readily with the cysteinyl sulfhydryl group in GSH to form GSH conjugates (Hiramatsu et al., 1990; Patel et al., 1991). Quinol-thioethers retain the ability to redox cycle and produce reactive oxygen species and arylate tissue macromolecules (Monks and Lau, 1998). Quinone-thioethers also inhibit enzymes that use GSH as a substrate, and, in particular, the 5-5-glutathionyl conjugates of dopamine and α-methyl dopa inhibit human GSH S-transferases (Ploemen et al., 1994).

Since there appears to be a transporter capable of transferring GSH and GSH conjugates from the circulation into the brain (Kannan et al., 1990), the systemic formation of GSH conjugates of α-MeDA and N-Me-α-MeDA, followed by uptake into and further metabolism by the brain, may provide a mechanism to explain the role of metabolism in MDA- and MDMA-mediated neurotoxicity. Uptake of 5-(glutathionyl-S-yl)α-MeDA into brain decreases in the presence of GSH, suggesting that the two compounds share the same transport mechanism (Miller et al., 1996). In contrast, inhibition of γ-GT with acivicin potentiates the uptake of 5-(glutathionyl-S-yl)α-MeDA into brain (Miller et al., 1996), and pretreatment of rats with acivicin potentiates both MDA- and MDMA-mediated long-term depletions in 5-HT and 5-HIAA (Bai et al., 2001). These data provide evidence that the serotoninergic toxicity observed following peripheral administration of MDA or MDMA requires the synthesis of metabolites that serve as substrates for γ-GT.

Materials and Methods

Animals. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing ~250 g were used for all in vivo experiments (n = 6–8 per experimental group). Animals were group-housed and maintained on a 12-h (8:00 AM to 8:00 PM) light/dark cycle, and food and water was provided ad libitum. Animals scheduled to undergo surgical cannula implantation were handled for ~2 weeks prior to surgery to reduce stress and discomfort. Following surgery, animals were individually housed and allowed a 5- to 6-day recovery period.

Synthesis of 5-(N-Acetyltyrtyl-S-yl)-N-methyl-α-methyltyrosine. 5-(N-Acetyltyrtyl-S-yl)-N-methyl-α-methyltyrosine was synthesized following an experimental procedure similar to that described by Miller et al. (1996) for 5-(N-acetyltyrtyl-S-yl)-α-MeDA. In short, 3 mg of tyrosinase from mushroom (EC 1.14.18.1.3, 2000 units/mg solid; Sigma-Aldrich, St. Louis, MO) in 200 μl of phosphate-buffered saline 50 mM, pH 7.4 buffer, 2.25 mg of N-acetyltyrtyl (Sigma-Aldrich) in 1000 μl of the phosphate-buffered saline buffer, and 3.25 μl of a methanolic solution of N-methyl-α-MeDA, equivalent to 2.5 mg (Pizarro et al., 2002) were incubated for 30 min in a shaker/water bath at 37°C (final reaction volume, 25 ml). The reaction was quenched with 2 ml of 5% formic acid. The reaction mixture was concentrated by lyophilization, and 5-(N-acetyltyrtyl-S-yl)-N-Me-α-MeDA was isolated by semipreparative reverse-phase HPLC (HPLC-UV; LC-6A; Shimadzu, Columbia, MD). The mobile phase was methanol and 1% acetic acid (15:85) with a flow rate of 5 ml/min, and the eluate was monitored at 225 nm; fractions corresponding to the compound of interest were collected and combined. Collected fractions were lyophilized, and the structure and purity of the compound were determined by HPLC-MS/MS and HPLC-CEAS. HPLC-MS/MS revealed a single compound with a molecular ion corresponding to 5-(N-acetyltyrtyl-S-yl)-N-Me-α-MeDA (M + 343.1). The molecular ion, once further fragmented, gives rise to several daughter ions, including those of N-acetyltyrtyl (m/z 161.9) and N-methyl-α-methyltyrosine (m/z 182.9).

Surgical Cannula Implantation. Guide cannulae were surgically implanted above the striatum as described previously (Duvall et al., 2000). Briefly, animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) supplemented with chloral hydrate (80 mg/kg i.p.). Atropine sulfate (250 μg/kg s.c.) was administered prophylactically to alleviate potential respiratory congestion. Animals were stereotaxically implanted with a unilateral guide cannula (21 g; Plastics One, Roanoke, VA). Cannulae were positioned 0.65 mm above the caudate putamen (anteroposterior, 0.2 mm; mediolateral, ± 3.0 mm; dorsoventral, 2.5 mm; Paxinos and Watson,
1997). To control for hemispheric differences, equal numbers of animals were implanted in the left or right hemisphere. Cannulae were fixed to the skull with dental acrylic and four stainless steel screws. Dummy cannulae were placed in the guide cannulas, and animals were individually housed and allowed a 5- to 7-day recovery period.

**In Vitro Recovery Calibration.** Microdialysis probes were of concentric design and were constructed in-house using PE 20 tubing as the inlet and fused silica (75 μm i.d.) within a 26-gauge shaft (Plastics One), with a 4-mm active membrane (mol wt. cutoff = 13,000 Da; Spectrum, Houston, TX). Prior to dialysis probe recovery, all probes were flushed with 70% ethanol and Nanopure water. At the day of recovery, 1.0-ml gas-tight 1000 series Hamilton syringes were filled with freshly prepared filtered Ringer’s solution (123.3 mM NaCl, 1.35 mM CaCl₂, 2.68 mM KCl, and 2.0 mM MgCl₂), pumped through the probe at 1.63 μl/min for 30 min, with the probe tips in a beaker containing the Ringer’s solution. To obtain probe recovery calibration, the Ringer’s solution was then replaced with Ringer’s solution supplemented with MDMA, 5-(glutathionyl)-MeDA, 2,5-bis(glutathionyl)-α-MeDA, and 5-(N-acetylcysteiny1ysyl)-N-Me-α-MeDA (20 nM) and maintained at 37°C. Two 20-min in vitro dialysis samples were collected, and dialysate was assayed by HPLC with electrochemical detection, and the average of the two samples was used as the percentage of recovery for each probe. Probe recovery was calculated by comparing the peak heights of each dialysate collected to those from prepared standards. The probes used in these experiments had recovery percentages ranging from 15 to 18%, and these values were used to correct for probe variability.

**Microdialysis Probe Implantation and Assay of Dialysate.** Microdialysis probes were lowered through the previously implanted guide cannulae into isoflurane or halothane anesthetized rats 12 to 15 h prior to the experiment and secured with dental acrylic. Probes were connected to a 1.0-ml gas-tight Hamilton 1000 series syringe mounted on a Model A syringe pump (Razel, Stamford, CT), and guide cannulae into isoflurane or halothane anesthetized rats 12 to 18%, and these values were used to correct for probe variability.

**Data Analysis and Statistics.** Concentrations of the thioether conjugates are presented as absolute values (pmol/10 μl) and expressed as the mean ± S.E. (n = 5–6). Where relevant, the Student’s t test was used to compare MDMA groups with MDMA + acivicin groups, and a p value of <0.05 was used to determine significance. Concentrations of monoamine neurotransmitters and their metabolites are presented as the percentage of decrease, and absolute values corrected again under the same condition, and 20-μl aliquots were used for HPLC analysis.

**High Performance Liquid Chromatography.** Thioether metabolite concentrations and monoamine levels were quantified by HPLC equipped with a four-channel coulometric electrode array system (HPLC-CEAS, ESA Inc., Chelmsford, MA) with electrode potentials set to +50, +150, +300, and +550 mV. 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 mM EDTA, 230 mM 1-octanesulfonic acid, and 5% methanol (pH 2.5). The flow rate was set at 1 ml/min. Quantification of the conjugate metabolites, 5-HT, dopamine, 5-HIAA, and 3,4-dihydroxyphenylacetic acid was achieved by comparing the peak area with standard curves generated from authentic standards.

**LC-MS/MS.** High-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) was performed on striatal dialysate samples with a Finnigan-MAT LCQ (Thermo Finnigan, San Jose, CA) electrospray ion-trap mass spectrometer coupled with a MAGIC 2002 microbore high-performance liquid chromatograph (Michrom BioResources, Auburn, CA). Dialysate (10 μl) was injected through the injector port, passed through a small molecular ion trap to remove salts, and eluted with 50% mobile phase A (acetonitrile/water/acetate acid/trifluoroacetic acid, 2:98:0.1:0.02) and 50% B (acetonitrile/water/acetate acid/trifluoroacetic acid, 90:10:0.09:0.02) at 20 μl/min. The eluate was directly analyzed by the LCQ using established protocols (Townsend et al., 2003). Automated data acquisition was carried out by data-dependent scanning with Finnigan Excalibur software (Thermo Finnigan). The scan event sequences included one full scan followed by 3 MS/MS of most intense ion in the parent ions list, which corresponds to the m/z of the thioether or N-acetyl metabolites (Table 1). Total run time was 6 min. The settings for the electrospray ionization were as follows: spray voltage, 3.5 kV; sheath gas and auxiliary gas flow rates, 60 and 5 ml/min, respectively; capillary temperature, 200°C; capillary voltage, 22 V; tube lens offset, 40 V. The electron multiplier was set at ~860 V; the scan time settings were performed with three microscans and 50 ms of maximum injection time for a full scan, and with five microscans and 200 ms of maximum injection time for an MS/MS scan. The target number of ions for MS was 1e8 and for MSn (n = 3) was 2e7. The full scan range for MS was 400 to 2000 Da. Data-dependent scanning was performed with an isolation width of 2 amu, a normalized collision energy level at 35%, an activation time of 30 ms, and a required minimal signal of 50,000 counts. Global-dependent data settings were as follows: reject mass width of 1 amu, exclusion mass width of 3 amu, dynamic exclusion enabled, repeat count of 2, repeat duration of 1 min, and exclusion duration of 1 min. Data were analyzed by comparing fragment patterns of known standards and experimental samples.

**Data Analysis and Statistics.** Concentrations of the thioether conjugates are presented as absolute values (pmol/10 μl) and expressed as the mean ± S.E. (n = 5–6). Where relevant, the Student’s t test was used to compare MDMA groups with MDMA + acivicin groups, and a p value of <0.05 was used to determine significance. Concentrations of monoamine neurotransmitters and their metabolites are presented as the percentage of decrease, and absolute values corrected again under the same condition, and 20-μl aliquots were used for HPLC analysis.

**TABLE 1**

<table>
<thead>
<tr>
<th>Mol. wt. and m/z ratios of α-MeDA and N-Me-α-MeDA thioethers used for LC-MS/MS analysis</th>
<th>5-(GSyl)-N-Me-α-MeDA</th>
<th>2.5-bis(GSyl)-N-Me-α-MeDA</th>
<th>5-(NAC)-N-Me-α-MeDA</th>
<th>2.5-bis(NAC)-N-Me-α-MeDA</th>
<th>5-(GSyl)-α-MeDA</th>
<th>2.5-bis(GSyl)-α-MeDA</th>
<th>5-(NAC)-α-MeDA</th>
<th>2.5-bis(NAC)-α-MeDA</th>
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</thead>
<tbody>
<tr>
<td>MOL. Wt.</td>
<td>486</td>
<td>791</td>
<td>342</td>
<td>503</td>
<td>472</td>
<td>777</td>
<td>328</td>
<td>489</td>
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<tr>
<td>m/z (MH⁺)</td>
<td>487</td>
<td>792</td>
<td>343</td>
<td>504</td>
<td>473</td>
<td>778</td>
<td>329</td>
<td>490</td>
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GSyl, glutathionyl-S-yl; NAC, N-acetylcysteiny1ysyl.
ues (picomoles per milligrams of tissue) are reported in the legends. Student's t test was used to compare control with treated groups. Results are considered significant at the $p < 0.05$ or $p < 0.01$. All Pearson correlation analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Student's Pearson correlation analysis was performed using GraphPad Prism reported as $r^2$.

**Results**

Brain dialysate samples collected from animals treated peripherally with MDMA or MDMA and acivicin were initially analyzed by tandem HPLC-MS/MS for the presence of thioether metabolites of N-Me-α-MeDA and α-MeDA and subsequently quantified by HPLC-CEAS. Fragmentation patterns with distinct similarities to those of synthetic standards were detected in the majority of MS/MS scans of animals treated with MDMA, providing initial evidence for the presence of MDMA and thioether metabolites in the brain. In particular, dialysate samples taken prior to drug administration (baseline) never produced MS/MS fragmentation patterns similar to those of authentic standards, indicating that MDMA administration was required to observe the MS/MS fragmentation profiles similar to those of the standards. As examples, the MS/MS of the MDMA parent ion (Fig. 1A, $m/z = 194.7$) yields fragments at $m/z$ 163 and 58 (Fig. 1B). These fragments were never detected in MS/MS analyses of a minor endogenous $m/z$ 194.2 ion present in dialysate samples taken prior to MDMA administration (Fig. 1C) but only following drug treatment (Fig. 1D). The LC-MS/MS of synthetic 2,5-bis(N-acetylcystein-S-yl)-N-Me-α-MeDA is illustrated in Fig. 2. The MS/MS of the 504 $M^+$ ion (Fig. 2A) produced a single major fragment at $m/z$ 405 (loss of $m/z$ 99; 57 from N-COCH3 and 42 from COO$^-$.). This fragment was absent in the MS/MS analysis of the endogenous $m/z$ 504 ion (Fig. 2B) but was a predominant fragment in the MS/MS spectra of the $m/z$ 504 ion present in the sample obtained from MDMA-treated rats (Fig. 2C). Additional fragments in the MS/MS spectra from the MDMA-treated sample may reflect differences in fragmentation characteristics between the two matrices (ACSF for treated samples). In this respect, the presence of Na$^+$ in ACSF would be expected to enhance the ionization (positive charge) of the compound, promoting further fragmentation and the appearance of additional $m/z$ ions in the LC-MS/MS analyses. The $m/z$ 447 would then be loss of $m/z$ 57 (the N-acetyl fragment).

**Quantitation of N-Me-α-MeDA-thioether Conjugates in Brain following Peripheral Administration of MDMA.** None of the metabolites were detected in any of the baseline (dialysate collected prior to drug administration) or control samples (s.c. saline). The kinetics of the elimination of 5-(glutathion-S-yl)-N-Me-α-MeDA and 2,5-bis(glutathion-S-yl)-N-Me-α-MeDA, and of the formation of 5-(N-acetylcysteine-S-yl)-N-Me-α-MeDA and 2,5-bis(N-acetylcystein-S-yl)-N-Me-α-MeDA (Fig. 3), are remarkably similar to the profiles obtained following i.c.v. administration of 5-(glutathion-S-yl)-α-MeDA (Miller et al., 1996). Maximum concentrations of 5-(glutathion-S-yl)-N-Me-α-MeDA and 2,5-bis(glutathion-S-yl)-N-Me-α-MeDA and subsequently quantified by HPLC-CEAS. Fragmentation patterns with distinct similarities to those of synthetic standards were detected in the majority of MS/MS scans of animals treated with MDMA, providing initial evidence for the presence of MDMA and thioether metabolites in the brain. In particular, dialysate samples taken prior to drug administration (baseline) never produced MS/MS fragmentation patterns similar to those of authentic standards, indicating that MDMA administration was required to observe the MS/MS fragmentation profiles similar to those of the standards. As examples, the MS/MS of the MDMA parent ion (Fig. 1A, $m/z = 194.7$) yields fragments at $m/z$ 163 and 58 (Fig. 1B). These fragments were never detected in MS/MS analyses of a minor endogenous $m/z$ 194.2 ion present in dialysate samples taken prior to MDMA administration (Fig. 1C) but only following drug treatment (Fig. 1D). The LC-MS/MS of synthetic 2,5-bis(N-acetylcystein-S-yl)-N-Me-α-MeDA is illustrated in Fig. 2. The MS/MS of the 504 $M^+$ ion (Fig. 2A) produced a single major fragment at $m/z$ 405 (loss of $m/z$ 99; 57 from N-COCH3 and 42 from COO$^-$.). This fragment was absent in the MS/MS analysis of the endogenous $m/z$ 504 ion (Fig. 2B) but was a predominant fragment in the MS/MS spectra of the $m/z$ 504 ion present in the sample obtained from MDMA-treated rats (Fig. 2C). Additional fragments in the MS/MS spectra from the MDMA-treated sample may reflect differences in fragmentation characteristics between the two matrices (ACSF for treated samples). In this respect, the presence of Na$^+$ in ACSF would be expected to enhance the ionization (positive charge) of the compound, promoting further fragmentation and the appearance of additional $m/z$ ions in the LC-MS/MS analyses. The $m/z$ 447 would then be loss of $m/z$ 57 (the N-acetyl fragment).
yl)-N-Me-α-MeDA (~35 and 43 pmol/10 μl dialysate) were reached between 20 to 40 min after s.c. injection of MDMA. In vitro recoveries were performed for each probe before it was used. In general, probes with recoveries in the 15 to 20% range were used. Thus, the total of ~78 pmol/10 μl dialysate represents an extracellular concentration of between 390 to 520 pmol/10 μl, or ~39 to 52 μM. If we assume a total brain volume of ~2 ml, then peak levels of between 78 to 102 nmol of 5-(glutathion-S-yl)-N-Me-α-MeDA are present in rat brain following a single s.c. injection of MDMA. Remarkably, these calculations fall within the range of the estimates made in an earlier study (Miller et al., 1996).

We have previously shown that pretreatment of animals with an inhibitor of γ-GT (acivicin) sharply increases (~7-fold) the uptake of 5-(glutathion-S-yl)-α-[3H]MeDA into brain (Miller et al., 1996). γ-GT is enriched in blood-brain barrier endothelial cells and catalyzes the first step in the metabolism of GSH and its S-conjugates. Inhibition of endothelial γ-GT may therefore enhance the delivery of 5-(glutathion-S-yl)-α-[3H]MeDA into brain by preventing its metabolic clearance at the blood-brain barrier, thereby increasing the pool of 5-(glutathion-S-yl)-α-[3H]MeDA available for the GSH transporter. Consistent with these findings, pretreatment of animals with acivicin prior to the subcutaneous administration of MDMA increased the concentrations of thioether metabolites of N-Me-α-MeDA in striatal dialysates (Fig. 4). In addition to increasing brain concentrations of the thioether metabolites of N-Me-α-MeDA, pretreatment with acivicin also potentiates MDMA and MDA-mediated neurotoxicity (Bai et al., 2001). Because some pharmacological manipulations may potentiate or protect against MDMA-mediated neurotoxicity by corresponding increases or decreases in MDMA-induced hyperthermia, we examined the effects of acivicin on MDMA and MDA-induced hyperthermia. When given alone, both MDMA and MDA produced the documented increase in body
temperature (rectal probe), but when given after acivicin treatment, neither MDMA nor MDA caused an increase in body temperature (Fig. 5). Thus, the ability of acivicin to potentiate MDMA and MDA induced neurotoxicity occurs within the context of an elimination of drug-induced hyperthermia.

**Correlation between Metabolite Concentration and Neurotoxicity.** There is a strong positive correlation between the concentration of the N-acetylcysteine metabolites in striatal dialysate and the degree of neurotoxicity. Seven days after dialysis, animals were euthanized, and brains were removed and dissected into multiple regions, and monoamine concentrations in tissue samples were analyzed by HPLC-CEAS. Interestingly, although strongest for the striatum, a positive Pearson correlation was observed between the concentration of mercapturates in the dialysate and decreases in both 5-HT (Fig. 6) and its metabolite 5-HIAA (data not shown) in the cortex, hippocampus, and hypothalamus. Thus, those animals that exhibited higher striatal concentrations of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA (Fig. 6) and 2,5-bis(N-acetylcystein-S-yl)-N-Me-α-MeDA (data not shown) consequently suffered greater decreases in brain 5-HT and 5-HIAA concentrations.

**Fig. 3.** Quantitation of thioether metabolites of N-Me-α-MeDA in striatal dialysate following s.c. administration of MDMA. Animals were pretreated with acivicin (18 mg/kg i.p.), and two 20-min baseline samples were collected before the animals were treated with MDMA (20 mg/kg s.c.). Dialysate samples were then collected every 20 min for 2 h and analyzed by HPLC-CEAS as described under Materials and Methods. A, time course of 5-(glutathion-S-yl)-N-Me-α-MeDA and 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA in dialysate. B, time course of 2,5-bis(glutathion-S-yl)-N-Me-α-MeDA and 2,5-bis(N-acetylcystein-S-yl)-N-Me-α-MeDA. Concentrations were determined by comparing the area under the curve of the metabolites in the samples to authentic standards, and the data are expressed as means ± S.E. (n = 6–8 per experimental group).

**Fig. 4.** Inhibition of γ-GT increases the concentration of thioether metabolites of MDMA in striatal dialysate. Animals were treated with MDMA (20 mg/kg s.c.) (C) or MDMA + acivicin (18 mg/kg i.p.) (●) and striatal dialysate was collected every 20 min for 2 h. Samples were analyzed by HPLC-CEAS as described under Materials and Methods. A, 5-(glutathion-S-yl)-N-Me-α-MeDA; B, 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA; C, 2,5-bis(glutathion-S-yl)-N-Me-α-MeDA, and D, 2,5-bis(N-acetylcystein-S-yl)-N-Me-α-MeDA. Concentrations were determined by comparing the area under the curve of the metabolites in the samples to authentic standards, and data are expressed as means ± S.E. (n = 6–8 per experimental group).
5-(N-Acetylcystein-S-yl)-N-Me-α-MeDA Is a Serotonergic Neurotoxicant. Intrastriatal administration of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA produced acute overt changes in behavior similar to that seen with MDMA and reported previously for thioether metabolites of N-Me-α-MeDA (Miller et al., 1995, 1997). 5-(N-Acetylcystein-S-yl)-N-Me-α-MeDA also significantly decreased striatal and cortical concentrations of 5-HT and 5-HIAA in a dose-dependent manner (Fig. 7). 5-HT and 5-HIAA concentrations in the hippocampus and hypothalamus of treated animals were also significantly lower than control levels, although these effects were substantially less than those occurring in the striatum and cortex. The data indicate diffusion of the hydrophilic mercapturate through the brain following injection into the striatum. Interestingly, 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA had a modest effect on the dopaminergic system. Thus, at the highest dose of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA (21 nmol) tested, dopamine concentrations in the striatum and cortex decreased ~16 to 18% from baseline levels (Fig. 8).

Discussion

HPLC-CEAS and HPLC-MS/MS analyses revealed that thioether metabolites of N-Me-α-MeDA are present in striatal dialysate of animals injected s.c. with MDMA (Figs. 1–3). This is the first time that neurotoxic metabolites of MDMA have been directly identified in the brain of animals receiving peripheral injections of the drug. Moreover, at least one of these metabolites, 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA, produces serotonergic neurotoxicity when injected directly into the striatum (Fig. 7). We conclude that the serotonergic neurotoxicity of MDMA (and MDA) is mediated, at least in part, by metabolism (O-demethylation) to N-Me-α-MeDA followed by oxidation of the catechol to the corresponding ortho-quinone and conjugation with GSH. This scenario is consistent with previous reports implicating a role for metabolism in MDMA-mediated serotonergic neurotoxicity (Gollamudi et al., 1989; Bai et al., 2001; Esteban et al., 2001; Monks et al., 2004).

Although metabolism has long been implicated in the serotonergic neurotoxicity of MDMA, identification of specific
5-HT and 5-HIAA in control animals were as follows: striatum 5-HIAA (B) concentrations in a dose-dependent manner. Absolute values for dopamine in the control group were as follows: striatum = 22.54 ± 0.97; cortex = 1.01 ± 0.26; hippocampus = 0.58 ± 0.13; and hypothalamus = 2.23 ± 0.47 pmol/mg tissue. A one-way analysis of variance followed by Student Newman-Keuls tests were conducted and data are expressed as the means ± S.E. (n = 6; *, p < 0.05).

Fig. 7. 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA is a neurotoxic metabolite of MDMA. HPLC-CEAS analysis was performed on tissue microdissected from several brain regions following intrastriatal injection of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA [7 (gray bars), 14 (white bars), or 21 (black bars) nmol, 4× at 12-h intervals]. 5-(N-Acetylcystein-S-yl)-N-Me-α-MeDA decreased dopamine concentrations in a dose-dependent manner. Absolute values for dopamine in the control group were as follow: striatum = 22.54 ± 0.97; cortex = 1.01 ± 0.26; hippocampus = 0.58 ± 0.13; and hypothalamus = 2.23 ± 0.47 pmol/mg tissue. A one-way analysis of variance followed by Student Newman-Keuls tests were conducted and data are expressed as the means ± S.E. (n = 6; *, p < 0.05).

Fig. 8. 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA causes a decrease in dopamine concentrations. HPLC analysis was performed on tissue from several brain regions, and 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA [7 (gray bars), 14 (white bars), or 21 (black bars) nmol, 4× at 12-h intervals] decreased dopamine concentrations in a dose-dependent manner. Absolute values for dopamine in the control group were as follow: striatum = 22.54 ± 0.97; cortex = 1.01 ± 0.26; hippocampus = 0.58 ± 0.13; and hypothalamus = 2.23 ± 0.47 pmol/mg tissue. A one-way analysis of variance followed by Student Newman-Keuls tests were conducted and data are expressed as the means ± S.E. (n = 6; *, p < 0.05).

Metabolites that reproduce this toxicity have been difficult. Inhibition of brain microvessel γ-GT activity with acivicin potentiates the serotonergic neurotoxicity of both MDMA and MDA (Bai et al., 2001), implicating the participation of metabolites that are substrates for this enzyme. It now appears that the ability of acivicin to potentiate the neurotoxicity of both MDMA and MDA occurs within the context of the ability of acivicin to abolish MDMA-induced hyperthermia (Fig. 5). The role of hyperthermia in MDMA-mediated neurotoxicity is a highly debatable issue and is coupled to the potential role of dopamine. Thus, although there is a substantial amount of evidence supporting a requirement for dopamine in MDMA-induced neurotoxicity, several investigators have discounted a direct relationship between dopamine and MDMA-mediated serotonergic neurotoxicity by suggesting that the role of dopamine is limited to its effect on raising ambient body temperature (Malberg et al., 1996, Colado et al., 1998; Malberg and Seiden, 1998; Yuan et al., 2001, 2002). For example, inducing hypothermia can protect against MDMA-mediated neurotoxicity (Malberg et al., 1996), and raising body temperature can potentiate the neurotoxicity. However, although pretreatment with fluoxetine provides protection against MDMA-induced serotonergic neurotoxicity, it did not inhibit MDMA-induced hyperthermia, indicating a temperature-independent mechanism (Falk et al., 2002). An essential role for hyperthermia in MDMA-induced neurotoxicity is further questioned by the finding that inhibiting monoamine oxidase-B with antisense oligonucleotides blocks the neurotoxicity of MDMA with no significant effect on ambient body temperature (Falk et al., 2002). Moreover, mazindol also protects against MDMA-induced neurotoxicity without altering MDMA-induced hyperthermia (Shankaran et al., 1999). Without doubt, the effects of ambient body temperature must be considered when interpreting data on MDMA-mediated neurotoxicity, because many of the chemicals that provide protection against MDMA-induced neurotoxicity also attenuate MDMA-induced hyperthermia; however, our own data reveals that we can actually potentiate MDMA-induced neurotoxicity in the absence of hyperthermia (Fig. 5). We share the opinion of Falk et al. (2002), who state that “increased body temperature can potentiate neurotoxic events, but the underlying basis of MDMA-induced neurotoxicity is unrelated to the production of hyperthermia”.

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The relative persistence of the mercapturates in brain is intriguing and indicates that these metabolites may accumulate in brain following multiple drug administration. This observation might provide some explanation to the fact that in most studies of MDMA-induced neurotoxicity, the preferred dosing regimen involves multiple MDMA doses over a short period of time rather than single doses. The former (multiple-dose) protocol more reliably produces neurotoxicity. Indeed, in monkeys that self-administer MDMA over several months, for a total drug exposure similar to a corresponding acute dosing protocol, no signs of neurotoxicity were observed (Fargettosi et al., 2004). In combination, the data suggest that a critical threshold concentration of neurotoxic metabolites must be reached to produce a permanent neurotoxic response, with such a threshold only achievable either at very high doses or after repeated dosing regimens over a relatively short period of time.

The mechanism by which metabolites of MDMA produce selective serotonergic neurotoxicity remains unclear; however, such metabolites are capable of reproducing some of the behavioral effects seen following MDA and MDMA administration in rats (Miller et al., 1995). The interesting aspect of this finding is that although the thiol conjugates produce behavioral responses akin to those of MDMA and MDA, α-MeDA did not. In other words, the presence of the amino acid moiety (either as the GSH tripeptide or as N-acetylcysteine) appears necessary to confer the neuropharmacological response, whereas the catecholamine moiety alone (α-MeDA) is insufficient for the response. Multiple roles for GSH (and cysteine) in the brain, other than its redox and cytoprotective functions, are being recognized (Shaw, 1998). Indeed, we have recently shown that 5-(glutathion-S-yl)-α-MeDA and 2,5-bis(glutathion-S-yl)-α-MeDA inhibit 5-HT transport into SK-N-MC cells transfected with the human 5-HT transporter (hSERT), with Kᵢ values about half of those of MDMA and MDA (Jones et al., 2004). In addition to inhibiting hSERT function, the metabolites also simultaneously stimulate the uptake of dopamine into hSERT-expressing cells (Jones et al., 2004). The neurotoxicity of MDMA and MDA might thus be a consequence of the metabolism-dependent stimulation of dopamine uptake into serotonergic neurons coupled to reactive oxygen species generation by the various redox active catechol-thioether metabolites and by dopamine.

The serotonergic neurotoxicity of 5-[(N-acetylcystein-S-yl)-N-Me-α-MeDA is relevant to the extrapolation of results obtained in animal models to humans. MDA is a minor metabolite of MDMA in humans, and the fraction of this metabolite available for O-demethylation to α-MeDA should also be low, thereby limiting its potential impact as a neurotoxicant. In contrast, N-Me-α-MeDA is a principal plasma metabolite of MDMA in humans (de la Torre et al., 2004). The fraction of N-Me-α-MeDA that undergoes oxidation to the ortho-quinone relative to the fraction that undergoes either conjugation with sulfate or glucuronic acid, or catechol O-methyltransferase (COMT) mediated O-methylation is not known. However, catechol estrogens undergo significant oxidation coupled to GSH conjugation (28–50%), even under conditions optimized for COMT-catalyzed O-methylation (Butterworth et al., 1996). It should be anticipated that similar reactions occur in humans because enzymes that participate in the activation of MDMA (CYP2D6) and inactivation of MDMA metabolites (COMT) are highly polymorphic in the human population. Thus, the fraction of N-Me-α-MeDA converted to neurotoxic metabolites may vary greatly in individuals exposed to similar doses of MDMA. Finally, species differences in the rate of O-demethylation of MDMA and MDA may underlie species differences in the neurotoxic response to these amphetamine analogs (Logan et al., 1988).

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References


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