SEROTONERGIC NEUROTOXIC METABOLITES OF ECSTASY IDENTIFIED IN RAT BRAIN.

By

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[3]Abbreviations: GSH, glutathione; γ-GT, γ-glutamyl transpeptidase; 5-HIAA, 5-hydroxyindoleacetic acid; HPLC-CEAS, HPLC-coulometric electrode array system; 5-HT, 5-hydroxytryptamine (serotonin); hSERT, human 5-HT transporter; icv, intracerebroventricular; LC-MS/MS, liquid chromatography-mass spectroscopy**;** α-MeDA, α-methydopamine MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine (ecstasy); N-Me-α-MeDA, N-methyl-α-methydopamine; ROS, reactive oxygen species.

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Abstract

The selective serotonergic neurotoxicity of 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) is dependent on their 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 LC-MS/MS and HPLC-CEAS 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-acetylcysteine 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-acetylcystein-S-yl)- N-methyl-α-MeDA and established that it is a relatively potent serotonergic neurotoxicant. Taken 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.

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Introduction:

Although the selectivity of (±)-3,4-methylenedioxymethamphetamine ([3]MDMA, ecstasy) and (±)-3,4-methylenedioxyamphetamine (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-demethylenated to 3,4-dihydroxymethamphetamine (N-methyl-α-methyldopamine, N-Me-α-MeDA) and 3,4-dihydroxyamphetamine (α-methyldopamine, 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 *ortho*-quinones and form adducts with glutathione (GSH) and other thiol-containing compounds (Lim et al. 1988, Hiramatsu et al., 1990). Alternatively, N-Me-α-MeDA and α-MeDA can be O-methylated to form 4-hydroxy-3-methoxymethamphetamine (3-O-Me-N-Me-α-MeDA) or 4-hydroxy-3-methoxyamphetamine (3-O-Me-α-MeDA) respectively.

There is a general consensus that MDA and MDMA neurotoxicity is dependent on metabolism of the parent drugs (de la Torre and Farré, 2004). Direct injection of MDA and MDMA into the brain fails to reproduce the acute or long-term neurotoxic effects evident following peripheral administration of these drugs (Molliver et al., 1986; Schmidt and Taylor, 1988; Schmidt et al., 1987; Paris and Cunningham, 1992) suggesting that systemic (liver) metabolism contributes to the neurotoxicity of MDA and MDMA. Indeed, differences between rats (resistant) and mice (sensitive) in their sensitivity to MDMA-mediated dopaminergic neurotoxicity, have been attributed to metabolic differences between the two species (Logan et al., 1988). In support of this view, inhibition of cytochrome P450 activity attenuates MDMA-mediated serotonergic neurotoxicity whereas stimulation of P450 activity potentiates both the

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metabolism and neurotoxicity of MDMA (Gollamudi *et al*., 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 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 serotonergic neurotoxicity. However, several known and putative metabolites of MDA and MDMA fail to produce 5-HT specific neurotoxicity after intracerebroventricular (icv) injection (McCann and Ricaurte, 1991; Elayan 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 ROS, and to arylate tissue macromolecules (Monks and Lau, 1998) Quinone-thioethers also inhibit enzymes which utilize GSH as a cosubstrate, and in particular, the 5-*S*-glutathionyl conjugates of dopamine and α-methyldopa 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-(glutathion-S-yl)-α-Me[^{3}H]DA into brain decreases in the presence of GSH, suggesting that the two compounds share the same transport mechanism (Miller et al., 1996). In contrast, inhibition of γ-GT with acivicin potentiates the uptake of 5-(glutathion-S-yl)-α-Me[^{3}H]DA 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 serotonergic toxicity observed following peripheral administration of MDA or MDMA requires the synthesis of metabolites that serve as substrates for γ-GT.

Consistent with these findings, intracerebroventricular (icv) injection of 5-(N-acetylcystein-S-yl)-α-MeDA and 5-(glutathion-S-yl)-α-MeDA into rats produces neurobehavioral changes characteristic of peripheral administration of MDMA/MDA, and acute increases in brain 5-HT and DA concentrations (Miller et al., 1996). Icv administration of 2,5-*bis*-(glutathion-S-yl)-α-MeDA (Miller et al., 1997) or direct injection of 5-(N-acetylcystein-S-yl)-α-MeDA or 5-(glutathion-S-yl)-α-MeDA (Bai et al., 1999), into the striatum, cortex or hippocampus, produces selective and prolonged depletions in 5-HT, and neurobehavioral changes similar to those caused by MDA and MDMA. In particular, 5-(N-acetylcystein-S-yl)-α-MeDA is an extremely potent serotonergic toxicant. However, neurotoxic metabolites of MDMA or MDA have yet to be identified in brain. Using *in vivo* microdialysis coupled to LC-MS/MS and HPLC-CEAS 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.

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Methods

Animals. Male Sprague-Dawley rats (Harlen Sprague-Dawley, Houston, TX) weighing ~250g were used for all *in-vivo* experiments (N = 6-8 per experimental group). Animals were group housed and maintained on a 12 hr (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 reduce stress and discomfort prior to surgery. Following surgery, animals were individually housed and allowed a 5-6 day recovery period.

Synthesis of 5-(N-acetylcystein-S-yl)-N-methyl-α-methyldopamine: 5-(N-acetylcystein-S-yl)-N-methyl-α-MeDA was synthesized following an experimental procedure similar to that described by Miller et al (1996) for 5-(N-acetylcystein-S-yl)-α-MeDA. In short, 3 mg of tyrosinase from mushroom enzyme (EC 1.14.18.1 3, 2000 units/mg solid, Sigma-Aldrich, St Louis, MO) in 200 μL, PBS 50 mM pH7.4 buffer, 2.25 mg N-acetylcysteine (Sigma-Aldrich, St Louis, MO) in 1000 μL, PBS 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 37ºC (final reaction volume 25 mL). The reaction was quenched with 2 mL 88% formic acid. The reaction mixture was concentrated by lyophilization and 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA was isolated by semi-preparative reverse phase (Beckman Ultrasphere ODS-5) HPLC (HPLC-UV, Shimadzu, LC-6A, Columbia, MD). The mobile phase was methanol and 1% acetic acid (15:85), flow rate 3 mL/min and the eluate was monitored at 225 nm and 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-acetylcystein-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-acetylcysteine (m/z 161.9) and N-methyl-α-methyldopamine (m/z 182.9).

Surgical cannula implantation. Guide cannulae were surgically implanted above the striatum as previously described (Duvauchelle *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). Cannulae were positioned 0.65 mm above the caudate putamen (AP: 0.2 mm; ML: ± 3.0 mm; DV: 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 5-7 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 ID) within a 26 gauge shaft (Plastics One, Roanoke), with a 4 mm active membrane (MW cutoff = 13,000 Da; Spectrum, Houston). Prior to dialysis probe recovery, all probes were flushed with 70% EtOH and Nanopure® water. At the day of recovery, 1.0 ml gastight 1000 series Hamilton syringes were filled with freshly prepared filtered Ringer's solution (128.3 mM NaCl, 1.35 mM $CaCl_2$, 2.68 mM KCl, and 2.0 mM $MgCl_2$), 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 Ringers solution was than replaced with Ringers solution supplemented with MDMA, 5-(glutathion-S-yl)-α-MeDA, 2,5-*bis*(glutathion-S-yl)-α-MeDA, and 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA (20 nM) and maintained at 37°C. Two twenty 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 percent 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 15-18% and these values were used to correct for probe variability and data were analyzed using the corrected values.

Microdialysis probe implantation and assay of dialysate: Microdialysis probes were lowered through the previously implanted guide cannulae into isofurane or halothane anesthetized rats 12-15 hours prior to the experiment, and secured with dental acrylic. Probes were connected to a 1.0 ml gastight Hamilton 1000 series syringe mounted on a syringe pump (Razel®, Model A), and freshly prepared Ringer's solution was pumped through the probe (1.63 μL). Animals implanted with the probe remained in a holding chamber (14 x 14 in) overnight with the syringe pump speed set at 0.261 μL/min. Bedding, food, and water were available in the holding chamber. Thirty min prior to the test session, the pump speed was changed to 1.63 μL/min. Dialysate samples were collected for 20 minutes prior to treatment with MDMA (baseline samples) and every 20 min (~32 μL) following MDMA administration (s.c.) for 2 hrs into 0.4 ml microcentrifuge tubes containing perchloric acid (0.5 N).

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Intrastriatal administration of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA. Guide cannulas (2g; Plastics One) were implanted into male Sprague-Dawley rats (AP: 0.2 mm; ML: ± 3.0 mm; DV: 4.5 mm; Paxinos and Watson, 1997) as described above for dialysis experiments. Following a 5-7 day recovery period the dummy cannula was replaced with PE20 tubing connected to a 1.0 ml gastight Hamilton 1000 series syringe containing various concentrations of 5-**(**N-acetylcystein-S-yl)-N-Me-α-MeDA in 8 μL Ringers solution and mounted on a syringe pump (Razel®, Model A). The drug solution was perfused into the striatum at a rate of 1.63 μL min for 5 minutes. Fresh Ringers solution was added to the syringe and perfusion continued for 10 min to ensure that all of the drug had been delivered to the brain. Following perfusion the dummy cannula was replaced and the animals were left undisturbed for 7 days prior to euthanasia.

Brain dissection and tissue preparation. Animals were euthanized by decapitation and their brains quickly removed and placed onto an ice-cold plate. Brains were dissected as to obtain brain regions enriched in 5-HT nerve terminals as previously described (Bai *et al.*, 1999), therefore, regions corresponding to the striatum, cortex, hippocampus, and hypothalamus were dissected free and frozen by liquid nitrogen. For neurotransmitter analyses, tissue was weighed and sonicated in ice-cold 0.1 N HClO4 containing 134 μM EDTA and 263 μM Na2S2O5 for 30s. The sonicated tissues were centrifuged at 13500*g* (4°C) for 10 min. Supernatants were centrifuged again under the same condition and aliquots (20 μL) 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 +350 mV. Sample aliquots were loaded onto an ESA HR-80 column (80 mm X

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. Quantitation of the conjugate metabolites, 5-HT, dopamine, 5-HIAA, and DOPAC was achieved by comparing the peak area with standard curves generated from authentic standards.

Tandem Mass Spectrometry (LC-MSMS). 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 injector port, passed through a small molecular ion trap to remove salts, and eluted with 50% mobile phase A (acetonitrile : water : acetic acid : trifluoroacetic acid, 2:98:0.1:0.02) and 50% B (acetonitrile : water : acetic acid : trifluoroacetic acid, 90:10:0.09:0.02) at 20 μL/min. The eluate was directly analyzed by LCQ using established protocol (Towndrow 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 are corresponding to the m/z of the thioether or N-acetyl metabolites (Table 1). Total run time was 6 min. The settings for the ESI were as follows: spray voltage, 3.5kV; sheath gas and auxiliary gas flow rates, 60 and 5 mL/min, respectively; capillary temperature, 200ºC; capillary voltage, 22 V; tube lens offset, 40V. The electron multiplier was set at –860V; the scan time settings were performed with 3 microscans and 50 msec of max injection time for full scan, and with 5 microscans and 200 msec of max infection time for MS/MS scan. The target number of ions for MS was 1e8 and for MSn was 2e7. The

full scan range for MS was 400-2000 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 msec, and a required minimal signal of 50,000 counts. Global dependent data settings were as follow, 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 fragmentation patterns of known standards and experimental samples.

Data Analysis and Statistics. Concentrations of the thioether and N-acetyl conjugates are presented as absolute values (pmol/10 μL) and expressed as the mean ± SE (n = 5-6). Where relevant, the Student's *t* test was used to compare MDMA groups to 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 percent decrease and absolute values (pmol/mg tissue) are reported in the legends. Student's *t* test was used to compare control to 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 t tests ($p < 0.05$; 0.01) were used to test the significance of the Pearson co-efficient (r; reported as r^2).

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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 (Figure 1A, m/z = 194.7) yields fragments at m/z 163 and 58 (Figure 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 (Figure 1C), but only following drug treatment (Figure 1D). The LC/MS/MS of synthetic 2,5-*bis*-(N-acetylcystein-*S*-yl)-N-Me-α-MeDA is illustrated in Figure 2. The MS/MS of the 504 MH^+ ion (Figure 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 (Figure 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 (Figure 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,

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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 adminstration) or control samples (sc 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-acetylcystein-*S*-yl)-N-Me-α-MeDA and 2,5-*bis*-(N-acetylcystein-*S*-yl)-N-Me-α-MeDA (Figure 3), are remarkably similar to the profiles obtained following icv 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 (~35 and 43 pmol/10 μL dialysate) were reached between 20-40 minutes after sc injection of MDMA. *In vitro* recoveries were performed for each probe before it was used. In general, probes with recoveries in the 15-20% range were utilized. Thus the total of ~78 pmol/10 μL dialysate represents an extracellular concentration of between 390-520 pmol/10 μL, or ~39-52 μM. If we assume total brain volume of ~ 2 mL, then peak levels of between 78-102 nmol of 5-(glutathion-*S*-yl)-N-Me-α-MeDA are present in rat brain following a *single* sc injection of MDMA. Remarkably, these calculations fall within the range of the estimates made in an earlier paper (Miller et al, 1996). Maximum concentrations of the mercapturates (~38 and 43 pmol/10 μL dialysate for 5-(N-acetylcystein-*S*-yl)-N-Me-α-MeDA and 2,5-*bis*-(N-acetylcystein-*S*-yl)-N-Me-α-MeDA respectively) were reached between 80-100 min, corresponding to combined extracellular concentrations similar to those of the corresponding GSH conjugates.

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Acivicin pretreatment increases concentrations of N-Me-α-MeDA-thioether conjugates in brain following peripheral administration of MDMA, and reduces MDMA-induced hyperthermia. 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-[^{3}H]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-[^{3}H]MeDA into brain by preventing its metabolic clearance at the blood-brain barrier, thereby increasing the pool of 5-(glutathion-*S*-yl)-α-[^{3}H]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 both N-Me-α-MeDA and α-MeDA in striatal dialysates (Figure 4). In addition to increasing brain concentrations of the thioether metabolites of N-Me-α-MeDA and 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 subsequent to acivicin treatment, neither MDMA nor MDA caused an increase in body temperature (Figure 5). Thus, the ability of acivicin to *potentiate* MDMA and MDA induced neurotoxicity occurs within the context of an elimination of drug-induced hyperthermia.

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Correlation between metabolite concentration and neurotoxicity. There is a strong positive correlation between the concentration of the N-acetylcysteine metabolites and the degree of neurotoxicity**.** Seven days after dialysis animals were euthanized, brains were removed, 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 (Figure 6) and its metabolite, 5-HIAA (data not shown) in the cortex, hippocampus and hypothalmus. Thus, those animals that exhibited higher striatal concentrations of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA (Figure 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.

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 as reported previously for thioether metabolites of 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 (Figure 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 ~16-18% from baseline levels (Figure 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 sc with MDMA (Figures 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 (Figure 7). We conclude that the serotonergic neurotoxicity of MDMA (and MDA) is mediated at least in part, by metabolism (O-demethylenation) 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 metabolites that reproduce this toxicity has 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 acivicin's ability to abolish MDMA-induced hyperthermia (Figure 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, Malberg and Seiden, 1998; Colado et al., 1998; Yaun 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 MAO-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, as 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 (Figure 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*".

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 (Fantegrossi 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 K_is 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 ROS 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-demethylenation 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-demethylenation of MDMA and MDA may underlie species differences in the neurotoxic response to these amphetamine analogs (Logan et al., 1988).

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Figure Legends

Figure 1: LC-MS/MS scans of authentic MDMA and MDMA in brain striatal dialysate samples. (**A**) Zoom scan of the full LC-MS of authentic MDMA in ACSF demonstrating the presence of the parent m/z 194.7 ion. (**B**) MS/MS scan of the parent ion (m/z 194) displaying fragmentation ions of m/z 163 and 58 characteristic of MDMA. (**C**) Average of 3 separate MS/MS spectra of the m/z 194 ion present in striatal dialysate prior to drug treatment (baseline), demonstrating no similarities in the fragmentation pattern with that of the MS/MS spectra of authentic m/z 194 from MDMA. (**D**) Average of 3 separate MS/MS spectra of the m/z 194 ion present in striatal dialysate 20 min after administration of MDMA (20 mg/kg; sc), exhibiting a fragmentation ions of m/z = 163 and 58 characteristic of the MDMA m/z 194.

Figure 2: LC-MS/MS analysis of the m/z 504 ion from (**A**) synthetic 2,5-*bis*(N-acetylcystein-S-yl)-Me-α-MeDA, and (**B**) striatal dialysate from untreated and (**C**) acivicin and MDMA-treated rats.

Figure 3: **Quantitation of thioether metabolites of N-Me-α-MeDA in striatal dialysate following sc administration of MDMA.** Animals were pretreated with acivicin (18 mg/kg, ip) and two 20 min baseline samples were collected before the animals were treated with MDMA (20 mg/kg; sc). Dialysate samples were then collected every 20 min for 2 hours and analyzed by HPLC-CEAS as described in the *Materials and Methods.* Panel (**A**) illustrates the time-course of 5-(glutathion-S-yl)-N-Me-α-MeDA and 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA in dialysate and panel (**B**) illustrates the 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 AUC

of the metabolites in the samples to authentic standards, and the data are expressed as means ± SE (N = 6-8 per experimental group).

Figure 4. Inhibition of γ-GT increases the concentration of thioether metabolites of MDMA in striatal dialysate. Animals were treated with MDMA (20 mg/kg; sc) (○) or MDMA + acivicin (18 mg/kg; ip) (●) and striatal dialysate collected every 20 min for 2 hrs. Samples were analyzed by HPLC-CEAS as described in the *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 AUC of the samples to known standards and data are expressed as means ± SE (N = 6-8 per experimental group).

Figure 5: Acivicin attenuates MDA and MDMA-mediated hyperthermia. Animals were treated with vehicle (control; ■), MDA (20mg/kg; ○), MDMA (20 mg/kg; ▲), acivicin (18 mg/kg; t△iangles), MDA + acivicin (●), or MDMA + acivicin; □) and rectal body temperature was measured every 30 min for 4 hours. *Significantly different from vehicle control and acivicin pretreated animals at $p < 0.05$.

Figure 6. **Concentrations of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA in striatal dialysate correlate with the extent of MDMA-mediated neurotoxicity**. Pearson correlation analysis was carried out on 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA concentrations in dialysate versus MDMA-mediated decreases in 5-HT concentrations. Correlation coefficients (r and r^2) are reported. Absolute values for 5-HT in the control group were as follows; (**A**) striatum = 2.37 ± 0.24 pmol/mg tissue, (**B**) cortex = 1.38 ± 0.22 pmol/mg tissue, (**C**) hippocampus = 1.51 ± 0.14

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pmol/mg tissue, and (**D**) hypothalamus = 4.02 ± 0.26 pmol/mg tissue. The average concentration of 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA in dialysate samples taken over the last three collection periods (60, 80 and, 120 min) were used to perform the correlation. Animals treated with MDMA alone (○) and animals treated with MDMA in the presence of acivicin (●). Student's t tests were preformed to test the significance of r/r^2 ($p < 0.05$, $p < 0.01$) using Graphpad prism

Figure 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 [grey bars], 14 [white bars], or 21 [black bars] nmol, 4X at 12 h intervals). 5-(N-Acetylcystein-S-yl)-N-Me-α-MeDA decreased 5-HT (**A**) and 5-HIAA (**B**) concentrations in a dose-dependent manner. Absolute values for 5-HT and 5-HIAA in control animals were as follows; striatum = 2.29 ± 0.21 and 2.01 ± 0.24; Cortex = 1.27 ± 0.19 and 0.97 ± 0.20; hippocampus = 1.45 ± 0.19 and 1.11 ± 0.23 pmol/mg tissue; and hypothalamus = 3.98 ± 0.22 and 1.54 ± 0.18 pmol/mg tissue, respectively. A one-way ANOVA followed by Student Newman-Kuels tests were conducted and data are expressed as the means ± SE (n = 6; $p < 0.05$ (*), $p < 0.01$ (†).

Figure 8. **5-(N-acetylcystein-S-yl)-N-Me-α-MeDA causes a decrease in dopamine concentrations**. HPLC analysis was preformed on tissue from several brain regions and 5-(N-acetylcystein-S-yl)-N-Me-α-MeDA (7 [grey bars], 14 [white bars], or 21 [black bars] nmol, 4X at 12 h intervals) decreased dopamine 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

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± 0.26; hippocampus = 0.58 ± 0.13; and hypothalamus = 2.23 ± 0.47 pmol/mg tissue. A one-way ANOVA followed by Student Newman-Kuels tests were conducted and data are expressed as the means ± SE (n = 6; *$p < 0.05$).

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Table 1:

Molecular weights (MW) and mass/charge (m/z) ratios of α-MeDA and N-Me-α-MeDA thioethers utilized for LC-MS/MS analysis.

	MW	**m/z**
5-(GSyl)-*N*-Me-α-MeDA	486	487
2,5-*bis*(GSyl)-*N*-Me-α-MeDA	791	792
5-(NAC)-*N*-Me-α-MeDA	342	343
2,5-*bis*(NAC)-*N*-Me-α-MeDA	503	504
5-(GSyl)-α-MeDA	472	473
2,5-*bis*(GSyl)-α-MeDA	777	778
5-(NAC)-α-MeDA	328	329
2,5-*bis*(NAC)-α-MeDA	489	490

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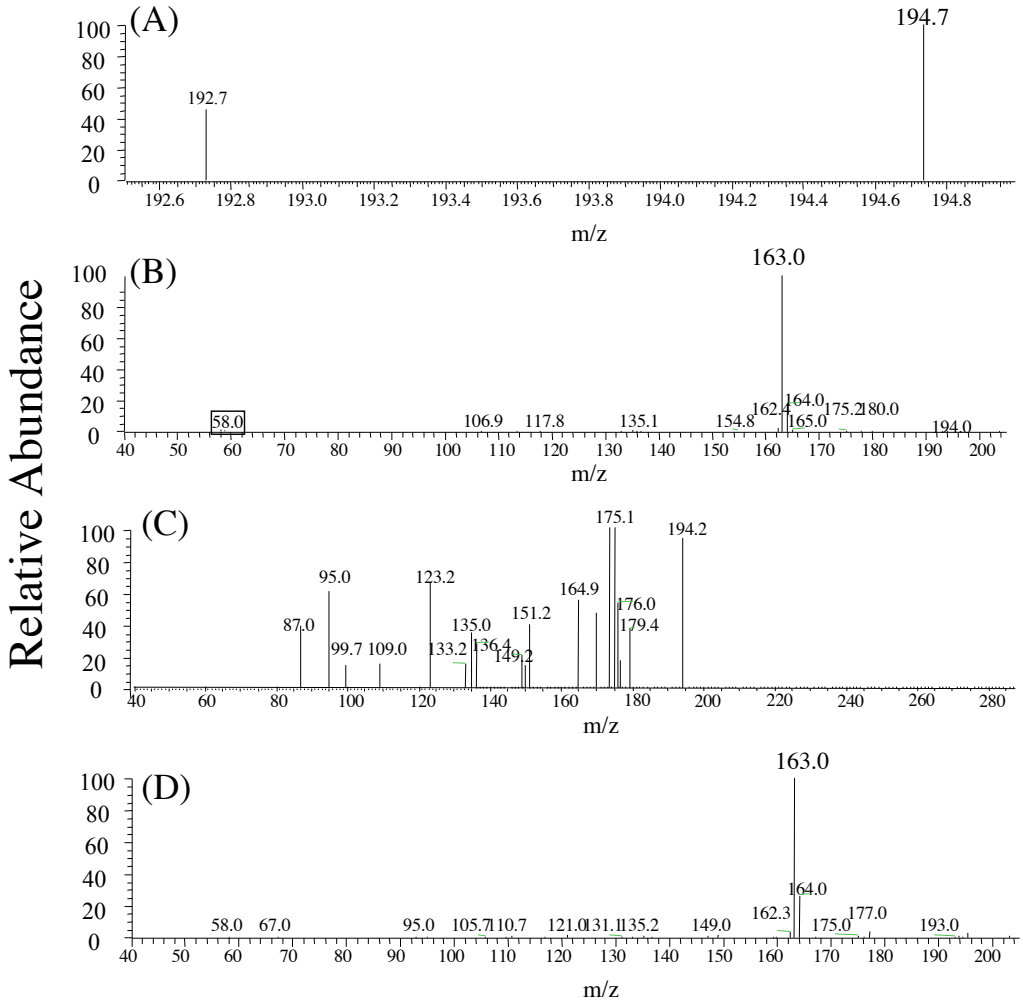

Figure 1

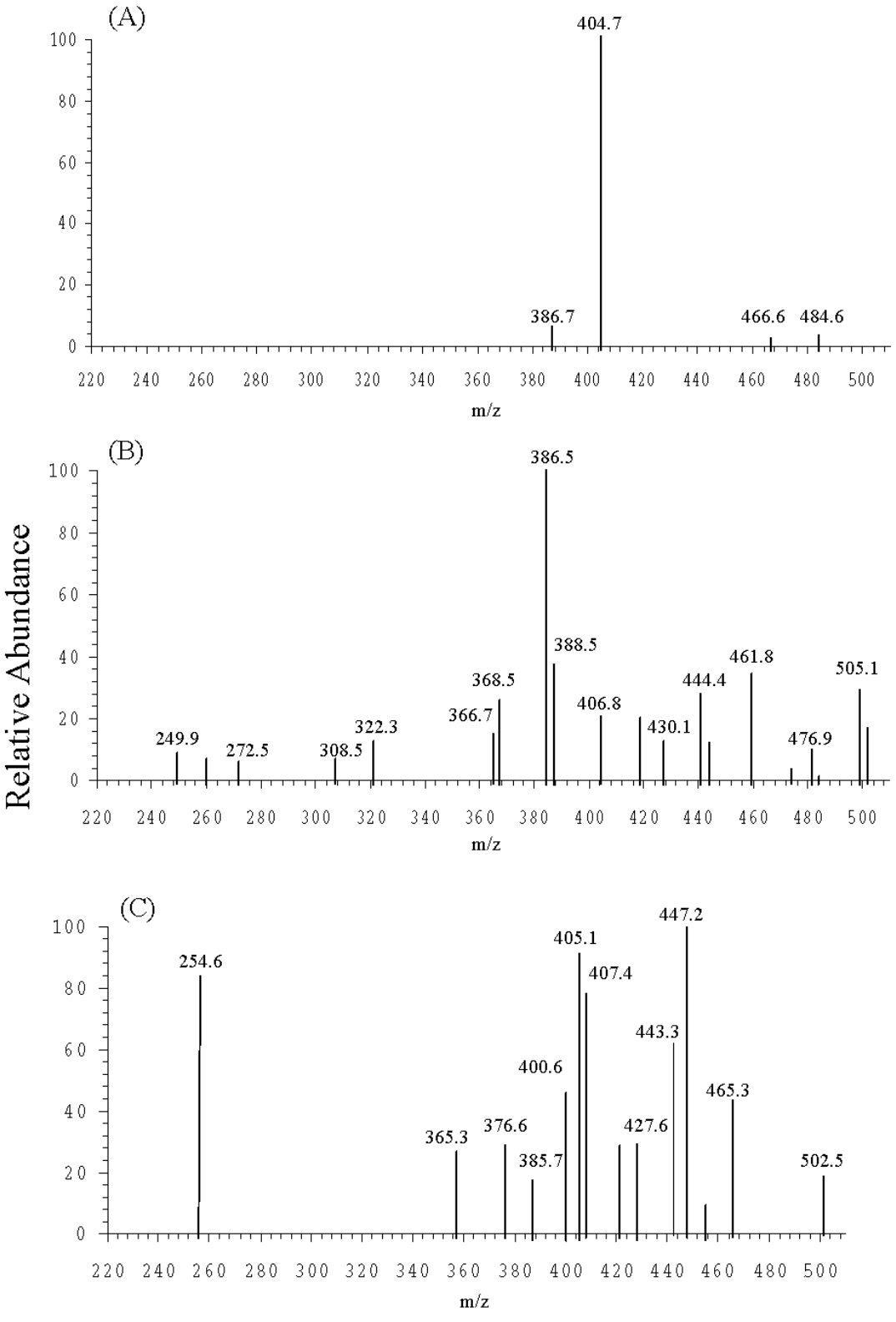
(A)
404.7
386.7
466.6
484.6
m/z
(B)
386.5
388.5
461.8
505.1
368.5
444.4
406.8
366.7
322.3
430.1
249.9
272.5
308.5
476.9
Relative Abundance
m/z
(C)
447.2
405.1
254.6
407.4
443.3
400.6
465.3
376.6
427.6
365.3
385.7
502.5
m/z

Figure 2

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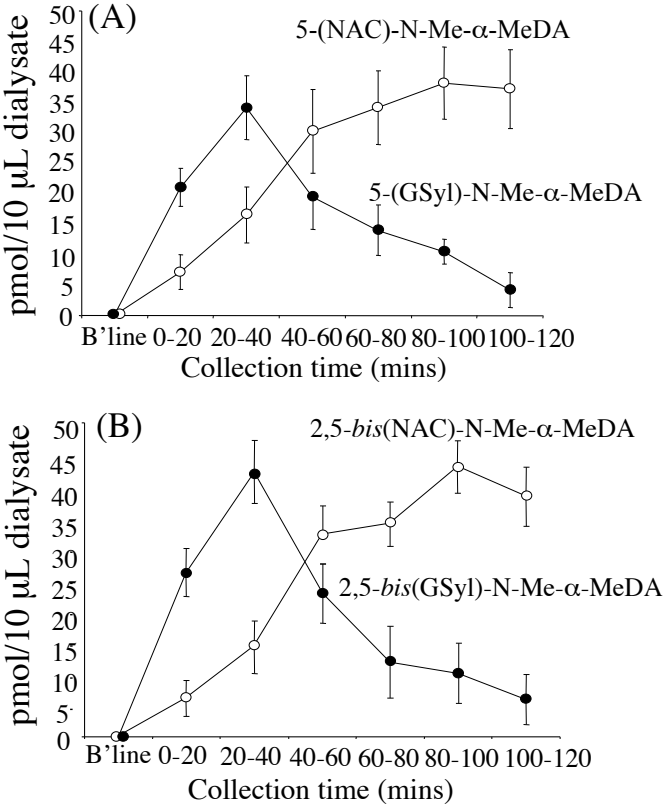

Figure 3

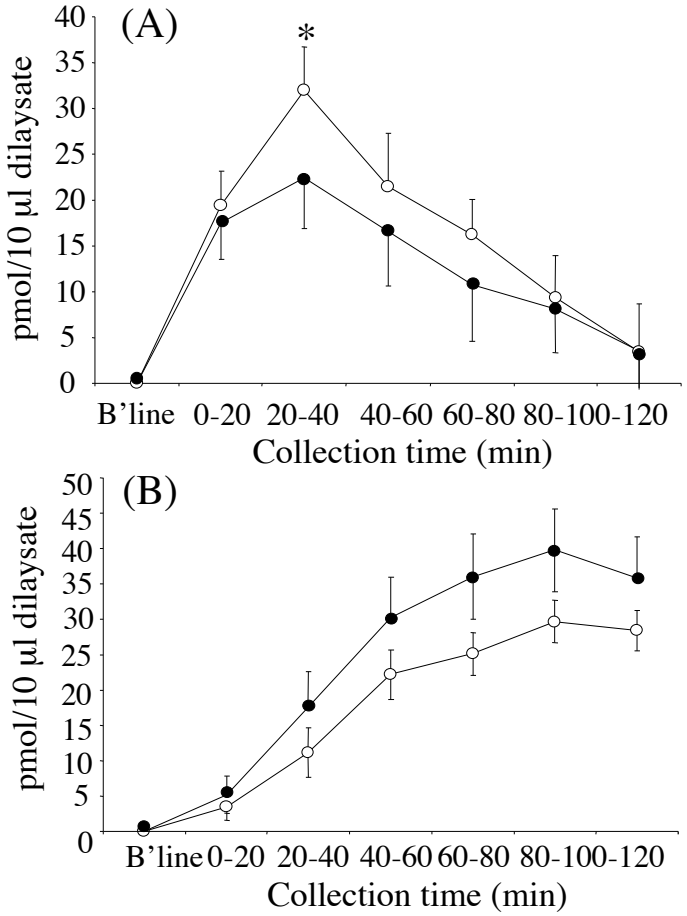

Figure 4 A&B

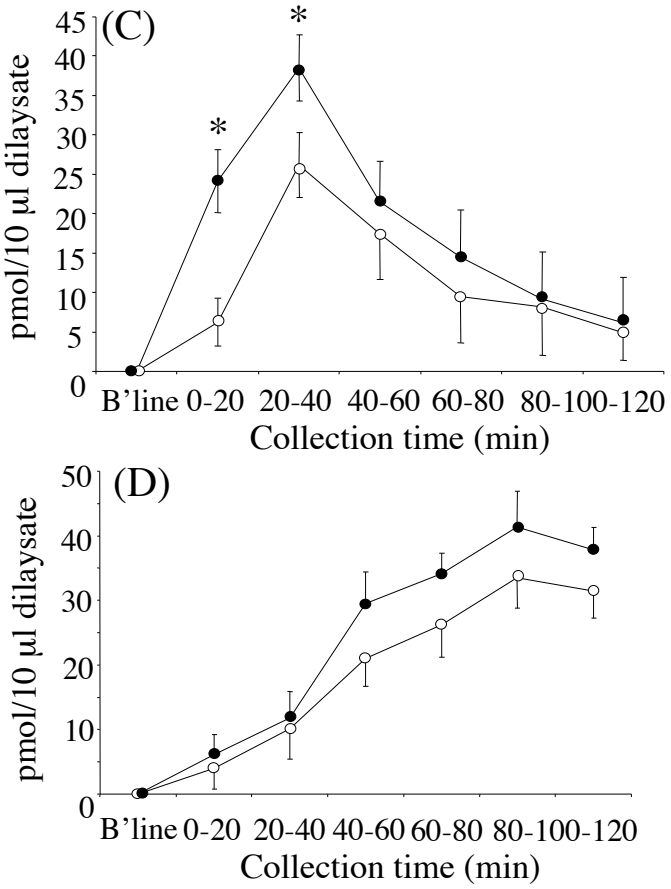

Figure 4 C&D

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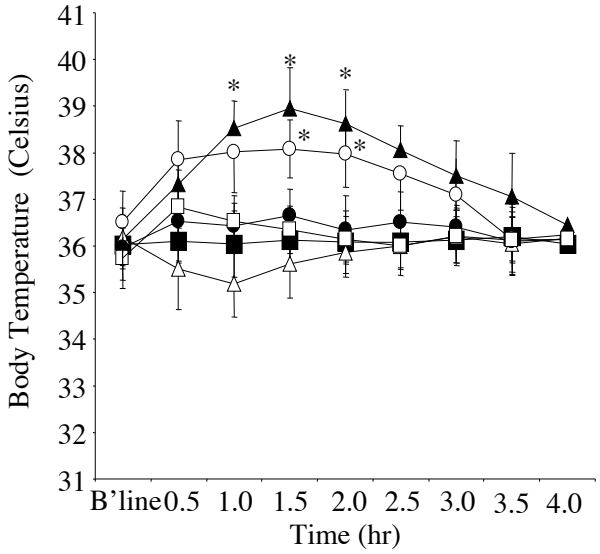

Figure 5

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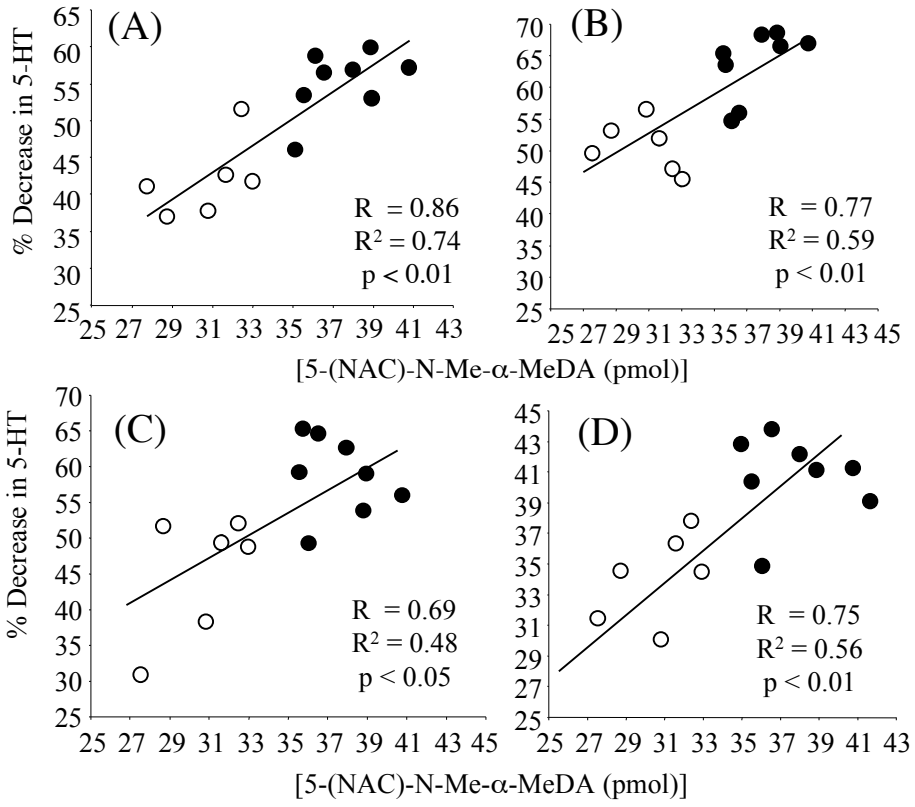

Figure 6

JPET Fast Forward. Published on January 5, 2005 as DOI: 10.1124/jpet.104.077628
This article has not been copyedited and formatted. The final version may differ from this version.

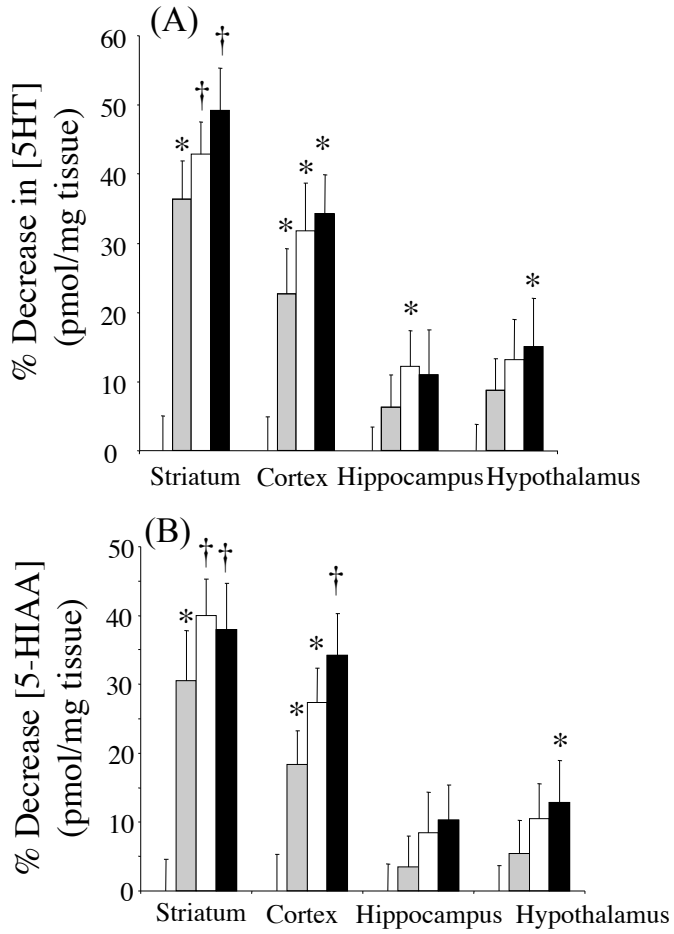

Figure 7

JPET Fast Forward. Published on January 5, 2005 as DOI: 10.1124/jpet.104.077628
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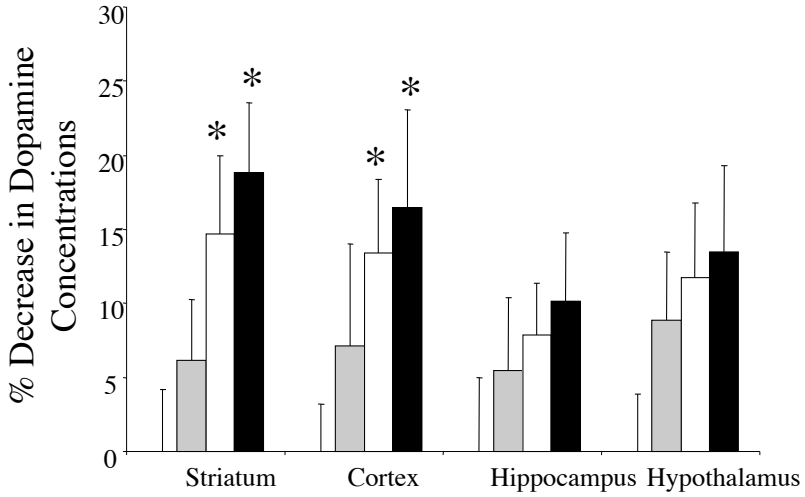

Figure 8