Catechol-O-Methyltransferase and 3,4-(±)-Methylenedioxyamphetamine Toxicity

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Metabolism of 3,4-(±)-methylenedioxyamphetamine (MDMA) is necessary to elicit its neurotoxic effects. Perturbations in phase I and phase II hepatic enzymes can alter the neurotoxic profile of systemically administered MDMA. In particular, catechol-O-methyltransferase (COMT) plays a critical role in determining the fraction of MDMA that is converted to potentially neurotoxic metabolites. Thus, cytochrome P450 mediated demethylation of MDMA, or its N-demethylated metabolite, 3,4-(±)-methylenedioxyamphetamine, give rise to the catechols, N-methyl-α-methyldopamine and α-methylidopamine, respectively. Methylation of these catechols by COMT limits their oxidation and conjugation to glutathione, a process that ultimately gives rise to neurotoxic metabolites. We therefore determined the effects of modulating COMT, a critical enzyme involved in determining the fraction of MDMA that is converted to potentially neurotoxic metabolites, on MDMA-induced toxicity. Pharmacological inhibition of COMT in the rat potentiated MDMA-induced serotonin deficits and exacerbated the acute MDMA-induced hyperthermic response. Using a genetic mouse model of COMT deficiency, in which mice lack a functional COMT gene, such mice displayed greater reductions in dopamine concentrations relative to their wild-type (WT) counterparts. Neither WT nor COMT deficient mice were susceptible to MDMA-induced decreases in serotonin concentrations. Interestingly, mice devoid of COMT were far more susceptible to the acute hyperthermic effects of MDMA, exhibiting greater increases in body temperature that ultimately resulted in death. Our findings support the view that COMT plays a pivotal role in determining the toxic response to MDMA.

Key words: 3,4-(±)-methylenedioxyamphetamine; catechol-O-methyltransferase; hyperthermia.

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
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<tr>
<td>CYP2D6</td>
<td>cytochrome P450 2D6</td>
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<td>DA</td>
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<td>DOPAC</td>
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<td>MDA</td>
<td>3,4-(±)-methylenedioxyamphetamine</td>
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<td>NE</td>
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<td>Ro 41-0960</td>
<td>2′-fluoro-3,4-dihydroxy-5-nitrobenzophenone</td>
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<td>VA</td>
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The neurotoxicity of 3,4-(±)-methylenedioxyamphetamine (MDMA) is well established (for review see Capela et al., 2009). In particular, metabolism of MDMA seems necessary for MDMA-induced neurotoxicity, as direct injections of MDMA into various compartments of the rodent central nervous system (CNS) fail to recapitulate the neurotoxicity observed following a systemic dose of MDMA (Escobedo et al., 2005; Esteban et al., 2001). Thioether metabolites of MDMA have been implicated in MDMA-induced neurotoxicity (Bai et al., 1999; Capela et al., 2007; Jones et al., 2005; Miller et al., 1997). These thioether metabolites have been identified in the CNS of rodents administered MDMA subcutaneously (Erives et al., 2008) as well as in the urine of humans after oral MDMA administration (Perfetti et al., 2009). Any perturbations in the metabolism of MDMA leading to changes in the amount of these thioether metabolites should modulate MDMA-induced neurotoxicity.

The important role of metabolism in MDMA-induced neurotoxicity has been further emphasized in a variety of pharmacological and genetic models, in which phase I and phase II enzyme inhibition/deficiency alters the metabolism of MDMA (Antolino-Lobo et al., 2010; Carmona et al., 2006; Colado et al., 1995). These studies indicate that inhibition/deficiency of cytochrome P450 2D6 (CYP2D6) or the rat homolog, CYP2D1, attenuates MDMA-induced serotonergic neurotoxicity, whereas inhibition of catechol-O-methyltransferase (COMT) potentiates neurotoxicity. As both CYP2D6 and COMT are polymorphi-
completely expressed, the possibility exists that an individual carrying a CYP2D6 ultrarapid metabolizer phenotype whilst simultaneously exhibiting impaired COMT function would be more susceptible to MDMA-induced neurotoxicity than an individual without such extreme genotypes. Indeed, the effects of polymorphisms in these genes, in humans, on MDMA kinetics, and cognitive function are clearly recognized (Cuyas et al., 2011; de la Torre et al., 2005; Schult et al., 2009). Such studies have emphasized the effects of CYP2D6/CYP2D1 on MDMA-induced neurotoxicity, whereas substantially less information is available on the effects of COMT inhibition on MDMA toxicity. Multiple single-nucleotide polymorphisms of COMT occur in humans (for review, see Tammimaki and Mannisto, 2010), the best-described being Val108/158Met. The allelic variant coding for methionine at position 108/158 bears a COMT protein that has significantly reduced catalytic activity in human postmortem tissue (Chen et al., 2004). Because COMT converts the MDMA metabolite, N-methyl-α-methylamphetamine (N-Me-α-MeDA), to the more stable 4-hydroxy-3-methoxyamphetamine (de la Torre et al., 2004), deficiencies in COMT activity could ultimately lead to greater formation of the thioether metabolites postulated to contribute to MDMA-induced neurotoxicity (Fig. 1).

Another important role of COMT is the termination of catecholamine neurotransmitter signaling, via the formation of inactive, methylated catechol metabolites (Eisenhofer et al., 1996). Individuals with a low activity COMT phenotype could, therefore, be prone to experiencing a greater duration or augmentation of the pharmacological effects of MDMA due to neurotransmitter release. One such concern is the potentiation of arguably the most detrimental acute effect of MDMA, hyperthermia (for review see Mills et al., 2004). The sympathetic nervous system is a critical player in thermogenesis, and neurotransmitters such as norepinephrine (NE), that play a role in thermogenesis, are released in greater quantities following MDMA administration (Wyeth et al., 2009). Coupled with the fact that COMT serves to inactivate NE, a low activity COMT phenotype individual could face severe risk of augmented hyperthermia.

Although pharmacological models of COMT inhibition have shown a potentiation of MDMA-induced neurotoxicity, no genetic models of COMT deficiency have yet been employed. The present study was undertaken to compare the effects of genetic and pharmacological deletion/inhibition of COMT on MDMA-induced neurotoxicity and hyperthermia.

MATERIALS AND METHODS

Drugs and chemicals. (±)MDMA-HCl was obtained from the National Institute on Drug Abuse (NIDA, Bethesda, MD). PCR reagents (Taq polymerase, MgCl2, 10× PCR buffer, and deoxynucleotide triphosphate mix) were purchased from New England BioLabs (Ipswich, MA). 2′-Fluoro-3,4-dihydroxy-5-nitrobenzophenone (Ro 41-0960) was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Animals. All procedures were reviewed and approved by the University of Arizona Institutional Animal Care and Use Committee. Adult female Sprague Dawley rats (250–300 g) were group housed and maintained on a 12 h light/dark cycle in a room maintained at 22 °C ± 1 °C. Food and water were provided ad libitum. Rats were allowed 1 week to acclimate to their surroundings before initiation of experiments. Three breeding pairs of COMT heterozygous mice (COMT+/−, background C57Bl/6) were kindly provided by the laboratories of Dr. Maria Karayiorgou and Dr. Joseph Gogos at Columbia University. These mice were bred to obtain sufficient numbers of animals to conduct statistically relevant experiments. Mice were housed on a 12 h light/dark cycle. Food and water were provided ad libitum. Mice were used in experiments once they reached 2.5 months of age. Mice were housed in a room maintained at 22 °C ± 1 °C, and all experiments were conducted at 22 °C ± 1 °C.

Genotyping. Genomic DNA was isolated from tail clippings using a standard Proteinase K digestion followed by high salt concentration/ethanol DNA precipitation. Genotyping was performed using the following primers: COMT F: ACC ATG GAG ATT AAC CCT GAC TAC G; Neomycin R: AGG TGA GAT GAC AGG AGA TC. Verification of COMT inhibition. To ensure Ro 41-0960 inhibited COMT, rats were pretreated with Ro 41-0960 or vehicle and euthanized 1.5 h later. Liver cytosolic and microsomal fractions were prepared following a method described by Fisher et al. (2009). Briefly, ~500 mg of liver tissue was homogenized in 5 ml of a solution comprised of 50 mM Tris-HCl, 1 mM EDTA, 154 mM KCl, pH 7.4 and centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was centrifuged at 100,000 × g for 60 min at 4 °C. Supernatant was collected as the cytosolic fraction, whereas the pellet was resuspended in a solution of 100 mM sodium pyrophosphate, 0.1 mM EDTA, pH 7.4 and centrifuged at 100,000 × g for 60 min at 4 °C. The pellet resulting from this centrifugation was resuspended in a solution of 10 mM potassium phosphate pH 7.4, 1 mM EDTA, and 20% (vol/vol) glycerol. Protein concentrations were determined using a Bio-Rad Protein Assay Reagent Kit (Bio-Rad Laboratories, Hercules, CA) as described by the manufacturer. Protein was used immediately (before storage at −80°C) in a COMT activity assay. COMT activity was determined using a method adapted from Kadowaki et al. (2005). Protein (200 μg) was incubated in a reaction mixture containing 4 mM MgCl2, and 2 mM
FIG. 1. Bioactivation of MDMA. MDMA can undergo N-demethylation in a reaction catalyzed by cytochrome P450 (CYP P450) enzymes to form methylenedioxyamphetamine. MDMA or MDA may undergo O-demethylation to form N-Me-α-MeDA or α-MeDA, respectively, via a reaction also catalyzed by various CYP P450 enzymes. These catechols may be acted upon by COMT to form 4-hydroxy-3-methoxy metabolites. The reactions catalyzed by COMT prevent oxidation of the catechols to quinones, and any subsequent thioether metabolites that may arise from the quinones. Abbreviations: MDMA, 3,4-(±)-methylenedioxymethamphetamine; MDA, 3,4-(±)-methylenedioxyamphetamine; N-Me-α-MeDA, N-methyl-α-methyldopamine; HMMA, 4-hydroxy-3-methoxy-methamphetamine; HMA, 4-hydroxy-3-methoxy-amphetamine; 5-(GSyl)-N-Me-α-MeDA, 5-(glutathion-S-yl)-N-methyl-α-methyldopamine; 5-(GSyl)-α-MeDA, 5-(glutathion-S-yl)-α-methyldopamine.

protocatechuic acid in 500 µl of 50mM Tris-HCl pH 7.4. Reactions were incubated at 37°C for 5 min before addition of S-adenosylmethylthionine (enough to bring S-adenosylmethionine concentration in the 300 ml reaction to 200µM). The complete reaction mixture was maintained at 37°C for 30 min before neutralizing the reaction by addition of 0.5 ml of ice-cold 0.5M perchloric acid. Mixtures were centrifuged at 15,000 × g for 10 min and 150 µl of the supernatant was then brought to neutral pH by addition of 37.5 µl of 1M NaOH and 37.5 µl of 0.5M Tris-HCl pH 7.4. Conversion of protocatechuic acid to vanillic acid (VA) was monitored by HPLC. A Shimadzu 10A system equipped with an ESA C-18, 3 µm, 4.6 × 80 mm column (Dionex, MA) was used to measure formation of VA. Mobile phase consisted of 80:20 water:methanol pH 2.5 at a flow rate of 0.5 ml/min. Absorbance was read at 260 nm. Samples were compared against a standard curve of VA to quantify amount formed.

Drug dosing. Pharmacological inhibition of COMT was achieved in rats by treatment with Ro 41-0960. Drug was dissolved in 60:40 DMSO:0.9% saline and administered to rats (40 mg/kg, ip). Rats dosed with either Ro 41-0960 or vehicle were then administered MDMA (20 mg/kg, sc) or saline 1.5 h after pretreatment. Mice received either MDMA (various dosing paradigms) or saline injections (sc). It was our intention to deliver the same dose of MDMA to COMT−/− mice as the dose delivered to wild-type (WT) and COMT+/− mice. However, the COMT−/− mice were far more sensitive to the acute toxicity of MDMA than either the WT or the COMT+/− animals, which necessitated the use of a number of different dosing paradigms. Therefore, male COMT−/− mice were treated with either 30 mg/kg × 1 (sc) or 15 mg/kg × 2 (sc, at a 6 h interval) MDMA and female COMT−/− mice were treated with 15 mg/kg × 3 (sc, at 3 h intervals) or 15 mg/kg × 2 (sc, at a 6 h interval) MDMA.

MMA, 4-hydroxy-3-methoxy-methamphetamine; HMA, 4-hydroxy-3-methoxy-amphetamine; 5-(GSyl)-N-Me-α-MeDA, 5-(glutathion-S-yl)-N-methyl-α-methyldopamine; 5-(GSyl)-α-MeDA, 5-(glutathion-S-yl)-α-methyldopamine.
**Tissue preparation for neurotransmitter analysis.** Animals were euthanized via CO₂ asphyxiation followed by decapitation, 1 week after administration of the final dose of MDMA. Brains were quickly excised and placed on a cold plate. The frontal cortex and striatum were dissected free of surrounding tissue and frozen in liquid nitrogen. Tissue was weighed and 10 volumes of ice-cold 0.1M perchloric acid containing 134μM EDTA and 263μM octanesulfonic acid sodium salt was added to the tissue. The tissue was then sonicated for 15 s followed by centrifugation at 16,000 g for 20 min at 4°C. Supernatant was filtered at 0.45 μm and used for neurotransmitter analysis via high-performance liquid chromatography coupled to electrochemical detection (HPLC-ECD). Neurotransmitter content was assayed using a Shimadzu 10ADvp system equipped with an ESA C-18, 3 μm, 4.6 × 80 mm column (Dionex) coupled to an ESA Model 5600A CoulArray system (Dionex). Mobile phase consisted of 35mM citric acid, 54mM sodium acetate, 324μM sodium-1-octanesulfonic acid, 171μM EDTA, 3% (vol/vol) methanol, 3% (vol/vol) acetonitrile, pH 4.0. Flow rate was 0.8 ml/min. Potentials were set at +50, +150, +300, and +450 mV. Injections onto the HPLC contained 50 μl of tissue preparation and peak areas were compared with a standard curve of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) to obtain neurotransmitter concentrations.

**Temperature measurement.** Rats or mice were anesthetized with isoflurane (5% in air to induce anesthesia and 1.5% for maintenance). A SubCue-Mini Datalogger (SubCue Dataloggers, Calgary, AB, Canada) was implanted in the peritoneal cavity. Temperature collection interval was set at 3 min.

**Plasma collection and preparation.** Naïve or MDMA-treated (30 mg/kg, sc) mice were used in this experiment. For MDMA-treated animals, 45 min after dosing mice were briefly anesthetized with isoflurane before a cervical dislocation was performed and whole blood was collected from the ventricle of the heart using a syringe containing 10 μl of 100 USP heparin. Whole blood was centrifuged at 3000 × g for 20 min at 4°C. Plasma supernatant was collected and stored at −80°C until preparation for HPLC-ECD analysis. For catecholamine extraction, plasma (100 μl) was added to an Eppendorf tube containing 30 mg of aluminum oxide (Wako Chemical USA, Richmond, VA), and 20 ng of an internal standard dihydroxybenzylamine hydrobromide, in 1.1 ml of 1.36M Tris buffer containing 9mM EDTA (pH 8.6). Tubes were shaken by hand for 10 min before centrifugation at 1000 × g for 1 min. Supernatant was discarded and the alumina pellet was washed with 1 ml of water. Tubes were then centrifuged at 1000 × g for 1 min to pellet alumina, which was washed with water and centrifuged twice more. The alumina pellets were transferred to a filter cup (UFC30GYV00, Millipore, Billerica, MA) in 500 μl water and centrifuged at 5000 × g for 10 min at 4°C. Water was discarded and alumina was immersed in 200 μl of 2% (vol/vol) acetic acid in 100μM EDTA for 10 min. After 10 min, the filter tube containing the alumina was centrifuged at 5000 × g for 10 min at 4°C. The resulting filtrate was analyzed by HPLC-ECD.

**HPLC-ECD measurement of plasma norepinephrine.** Plasma NE concentrations were measured using a Shimadzu 10ADvp system with an Eicom P3-CA-PC precolumn in-line before an Eicom CA-5ODS, 2.1 × 150 mm separation column (Eicom, San Diego, CA). The HPLC was coupled to an Eicom ECD-700 electrochemical detector (Eicom). Mobile phase consisted of 75.9mM NaH₂PO₄, 12.1mM Na₂HPO₄, 2.8mM sodium-1-octanesulfonic acid, 134μM EDTA, and 12% (vol/vol) methanol, pH 6.0. Flow rate was 0.18 ml/min. Working electrode was set at +450 mV. Plasma preparations (50 μl) were injected onto the HPLC and NE concentrations were determined by comparison against a standard curve.

**Statistics.** Concentrations of neurotransmitters are presented as scatter plots, where each point represents the absolute value of neurotransmitter measured (pmol/mg tissue). The mean of each treatment group is represented by a horizontal bar in the corresponding column. Neurotransmitter analysis of brain tissue and the COMT activity analysis were assayed by one-way ANOVA followed by Tukey post hoc tests. Plasma NE analysis and body temperature analyzes were assayed by two-way ANOVA followed by Bonferroni’s post hoc tests. A p-value <0.05 was used as the significance threshold. All analyses were performed using GraphPad Prism 5 software (La Jolla, CA). All data presented in the body of this manuscript are given as the mean ± standard error.

**RESULTS**

**Ro 41-0960 Inhibits COMT in Cytosolic and Microsomal Fractions of Rat Liver**

Protocatechuic acid is a substrate for COMT, the latter catalyzing a reaction that yields VA. Analysis of VA formation revealed significant differences between rats dosed with Ro 41-0960 and those dosed with vehicle control (Fig. 2). Cytosolic liver fractions prepared from rats dosed with 40 mg/kg (ip) Ro 41-0960 exhibit approximately 10% of the COMT activity (0.017 ng/min/μg protein) measured in vehicle-treated rats (0.166 ng/min/μg protein). The membrane-bound form of COMT proved less efficient in this experimental reaction, as microsomes prepared from vehicle-treated rats formed VA at a rate of 0.006 ng/min/μg protein, only ~4% of the rate seen in cytosolic fractions. Formation of VA in the microsomal fractions prepared from Ro 41-0960 treated rats were below the detection limits of the method.
**FIG. 2.** Ro 41-0960 inhibits hepatic COMT. Cytosolic and microsomal fractions of liver from rats treated with either Ro 41-0960 (40 mg/kg, ip) or vehicle were prepared and assayed for COMT activity as described in the Materials and Methods section. Each symbol represents the average from two HPLC injections of a single hepatic preparation. The mean is represented by a horizontal bar in each treatment group. *p < 0.05 compared with vehicle treated control of same hepatic preparation.

**FIG. 3.** COMT inhibition potentiates MDMA-induced neurotoxicity in rat. Rats pretreated with Ro 41-0960 before receiving MDMA display greater deficits in serotonin content than vehicle + MDMA animals. Each symbol represents the value obtained from a single animal and the mean for each group is represented by a horizontal bar. *p < 0.05 compared with vehicle + MDMA group in identical brain region.

**Pharmacological Inhibition of COMT in Rat Potentiates MDMA-Induced Neurotoxicity**

Rats pretreated with Ro 41-0960 (40 mg/kg, ip) followed by MDMA (20 mg/kg, sc) exhibited a greater depletion of 5-HT in areas of the brain heavily innervated with serotonergic nerve terminals (Fig. 3). In the frontal cortex, neurotransmitter analysis revealed that animals pretreated with Ro 41-0960 prior to MDMA had ~40% less 5-HT than those animals pretreated with vehicle followed by MDMA. Similar findings were discovered in the striatum (~45% reduction) and hippocampus (~65% reduction). A statistically significant difference was reached in the cortex and striatum, though not in the hippocampus when compared against vehicle + MDMA treated animals. The results are consistent with prior studies (Antolino-Lobo et al., 2010). A similar trend was noticed with respect to the 5-HT metabolite, 5-HIAA (data not shown). Thus, animals receiving Ro 41-0960 pretreatment exhibited a 20, 33, and 40% reduction in 5-HIAA concentrations in the cortex, striatum, and hippocampus, respectively. DA and DOPAC levels remained unchanged in the striatum across treatment groups (data not shown).

**FIG. 4.** MDMA-induced hyperthermia is exacerbated in COMT inhibited rats. Rats were pretreated with either vehicle (open symbols) or Ro 41-0960 (closed symbols) followed by either MDMA (squares) or saline (circles). Each symbol represents the average temperature of three rats at 15 min time intervals. The vertical dashed line represents time of MDMA or saline administration. *p < 0.05 between vehicle + saline and vehicle + MDMA treated groups. †p < 0.05 between Ro 41-0960 + saline and Ro 41-0960 + MDMA treated groups. §p < 0.05 between vehicle + MDMA and Ro 41-0960 + MDMA treated groups.

**MDMA-Induced Hyperthermia**

Rats treated with a single dose of MDMA (20 mg/kg, sc) exhibit an increase in core body temperature (Fig. 4). This effect was most pronounced in those rats pretreated with Ro 41-0960 where average peak body temperature reached 41.06°C ± 0.28°C versus rats administered vehicle prior to MDMA administration, where average peak body temperature reached 39.86°C ± 0.33°C. The average peak body temperature in vehicle treated rats was 38.71°C ± 0.10°C. No significant temperature elevations were observed in rats without MDMA treatment. Statistically significant differences were revealed when MDMA treated rats were compared against appropriate control groups, and when the Ro 41-0960 + MDMA treated rats were compared against the vehicle + MDMA treated rats (Fig. 4).

**MDMA-Induced Deficits in Neurotransmitter Levels are Potentiated in a Genetic Model of COMT Depletion**

Generally, both COMT+/- and COMT−/− mice dosed with MDMA were more sensitive to MDMA-induced neurotoxicity than their WT counterparts (Figs. 5 and 6), with few exceptions. Neurotransmitter analysis of the striatum of female COMT+/- mice dosed with MDMA (30 mg/kg × 3, sc, at 3 h intervals) revealed a 65% decrease in DA content compared with female COMT+−/− dosed with saline (Fig. 6A). In WT female counterparts, this deficit was 35%. Male COMT+/- mice administered MDMA (30 mg/kg × 3, sc, at 3 h intervals) exhibited a striatal DA deficit of 35% compared with male
FIG. 5. Genetic COMT deficiency in male mice leads to exacerbation of MDMA-induced dopaminergic neurotoxicity. A number of different dosing paradigms were used with the male mice (30 mg/kg × 3, black symbols; 30 mg/kg × 1, gray symbols; and 15 mg/kg × 2, red symbols). Various brain regions were assayed for neurotransmitters. Striatal DA (A), DOPAC (B), and 5-HT (C), as well as cortical 5-HT (D) were investigated. Each symbol represents the value obtained from a single animal and the mean for each group is represented by a horizontal bar. *p < 0.05 as compared with saline treated controls of identical genotype. †p < 0.05 as compared with WT mice of identical dosing paradigm. Abbreviations: WT, wild-type (COMT+/+); Het, heterozygous (COMT+/−); KO, knock-out (COMT−/−).

COMT+/− mice receiving saline (Fig. 5A). In WT males, this deficit was approximately 45%.

DOPAC levels in the COMT+/− mice followed a similar pattern to that of DA (Figs. 5B and 6B). COMT+/− mice showed slightly elevated levels of basal DOPAC concentrations in the striatum (9.05 ± 0.37 pmol/mg tissue in females and 7.79 ± 0.13 pmol/mg tissue in males) compared with WT counterparts (7.00 ± 0.31 pmol/mg tissue in females and 6.25 ± 0.22 pmol/mg tissue in males). Female COMT+/− mice administered MDMA displayed a 53% decrease in DOPAC levels compared with saline treated COMT+/− mice, whereas in WT females, this deficit was only 20%. Male COMT+/− mice treated with MDMA exhibited a 20% decrease in striatal DOPAC as compared with COMT+/− saline controls, but in WT males, this deficit reached 30%.

5-HT concentrations remained largely unchanged in WT and COMT+/− mice in both regions of the brain that were assayed (Figs. 5C and D, 6C and D). Only in female COMT+/− mice did 5-HT concentrations decrease more than 10% (11% in cortex and 13% in striatum) following MDMA administration. Only COMT+/− females administered MDMA (30 mg/kg × 3, sc, at 3 h intervals) revealed a statistically significant difference when absolute 5-HT concentrations were compared against WT females treated with the same MDMA regimen.

MDMA administered to female COMT−/− mice was lethal in three of five animals dosed at 30 mg/kg × 1 (sc), and lethal in five of nine animals dosed at 15 mg/kg × 3 (sc, at 3 h intervals). However, all female COMT−/− mice survived the 15 mg/kg × 2 (sc, at a 6 h interval) dosing regimen. Male COMT−/− mice were even more susceptible to acute MDMA toxicity; a dose of 30 mg/kg × 1 (sc) being lethal in 9 of 10 animals, and lethal in 16 of 19 animals dosed at 15 mg/kg × 2 (sc, at a 6 h interval).

Female COMT−/− mice dosed with MDMA at 15 mg/kg × 3 (sc, at 3 h intervals) exhibited a 55% decrease in DA content compared with COMT−/− females treated with saline (Figs. 5A and 6A). Female WT mice administered the identical dose of MDMA exhibited a 33% decrease in DA compared to their saline counterparts. Female COMT−/− mice dosed with MDMA at 15 mg/kg × 2 (sc, at a 6 h interval) displayed a 15% decrease in striatal DA content compared with their saline counterparts. Female WT mice administered MDMA at 15 mg/kg × 2 (sc, at a 6 h interval) showed no DA deficits relative to their WT saline counterparts. Male COMT−/− mice dosed with MDMA at 15 mg/kg × 2 (sc, at a 6 h interval) showed only a 5% decrease in striatal DA content, whereas WT mice receiving an identical dose of MDMA exhibited no deficits in DA concentrations. The only male COMT−/− mouse surviving the single 30 mg/kg MDMA administration displayed an
Genetic COMT deficiency in female mice leads to exacerbation of MDMA-induced dopaminergic neurotoxicity. A number of different dosing paradigms were used with the female mice (30 mg/kg x 3, black symbols; 15 mg/kg x 3, gray symbols; and 15 mg/kg x 2, red symbols). Various brain regions were assayed for neurotransmitters. Striatal DA (A), DOPAC (B), and 5-HT (C), as well as cortical 5-HT (D) were investigated. Each symbol represents the value obtained from a single animal and the mean for each group is represented by a horizontal bar. *p < 0.05 as compared with saline treated controls of identical genotype. †p < 0.05 as compared with WT mice of identical dosing paradigm. Abbreviations: WT, wild-type (COMT+/+); Het, heterozygous (COMT+/−); KO, knock-out (COMT−/−).

80% decrease in striatal DA content compared with saline controls, whereas this dose proved insufficient to cause any DA depletion in WT mice.

Basal levels of DOPAC in the striatum were significantly elevated in COMT−/− animals (17.75 ± 0.48 pmol/mg tissue in females and 17.17 ± 0.48 pmol/mg tissue in males) compared with their WT counterparts (7.00 ± 0.31 pmol/mg tissue in females and 6.25 ± 0.22 pmol/mg tissue in males, Figs. 5B and 6B). Female COMT−/− mice exhibited a 33% decrease in striatal DOPAC following a 15 mg/kg x 3 (sc, at 3 h intervals) MDMA dosing regimen and a 15% decrease on the 15 mg/kg x 2 (sc, at 6 h interval) dosing regimen. These deficits were 36% and 9% in WT females, respectively. Male COMT−/− mice exhibited a 13% DOPAC reduction on the 15 mg/kg x 2 (sc, at 6 h interval) MDMA dosing regimen. The lone surviving male given a single dose of MDMA at 30 mg/kg exhibited a 67% reduction in DOPAC. These DOPAC deficits in male WT mice were 0 and 13%, respectively.

5-HT concentrations in both the frontal cortex and striatum were largely unaffected by all MDMA regimens in all three genotypes (Figs. 5C and D, 6C and D), with no significant differences noted between any MDMA dose tested and saline treated animals of appropriate genotype. However, a statistically significant difference was found between the absolute 5-HT concentrations from COMT−/− males of the 15 mg/kg x 2 MDMA (sc, at a 6 h interval) dosing regimen and WT males treated with the same MDMA regimen, as well as COMT+/− females of the 30 mg/kg x 3 MDMA (sc, at a 3 h interval) dosing regimen and WT females of the identical MDMA treatment regimen.

MDMA Induces Hyperthermia in COMT−/− Mice But Not in Their WT Counterparts

Only COMT−/− mice exhibited an increase in core body temperature following a single dose of MDMA (30mg/kg, sc, Fig. 7). Basal temperatures in all animals averaged 36.56°C ± 0.10°C. Average peak body temperature in the male COMT−/− mice reached 40.18°C ± 0.27°C. Female COMT−/− mice reached an average peak body temperature of 38.84°C ± 0.88°C. WT animals displayed no marked temperature elevations. All male COMT−/− mice exhibited a similar temperature pattern; initially a state of hyperthermia that persisted for approximately 3 h which subsequently transitioned rapidly into a state of hypothermia until death approximately 6 h after MDMA administration. Female COMT−/− exhibited a more varied temperature response. Two of the female COMT−/− mice displayed no temperature changes following MDMA administration, whereas the remaining three females responded in much the same fashion as the males—an initial state of hyperthermia followed by hypothermia and, eventually,
death. Thus, only male COMT−/− mice displayed statistically significant differences in body temperature versus WT counterparts.

*Increased Plasma Norepinephrine in COMT−/− Mice Treated with MDMA*

NE plasma concentrations in COMT−/− mice were greater than those in WT mice for both genders, but only reached statistically significant differences in male mice (Fig. 8). However, the percentage increase of plasma NE 45 min after MDMA administration was similar in WT and COMT−/− mice. In COMT−/− females, a single dose of MDMA (30 mg/kg, sc) produced a 181% increase in plasma NE (2.59 ± 0.35 ng/100 μl plasma to 4.68 ± 1.57 ng/100 μl plasma, Fig. 8B). The increase in NE concentrations in WT female mice was 275% (0.83 ± 0.21 ng/100 μl plasma to 2.29 ± 0.28 ng/100 μl plasma). A single dose of MDMA (30 mg/kg, sc) caused a 265% increase in plasma NE (1.77 ± 0.26 ng/100 μl plasma to 4.49 ± 0.49 ng/100 μl plasma) in male COMT−/− mice, and a 237% increase (0.80 ± 0.35 ng/100 μl plasma to 1.90 ± 0.73 ng/100 μl plasma) in WT males (Fig. 8A).

**DISCUSSION**

Systemically administered MDMA produces serotonergic deficits in rats and nonhuman primates, in addition to producing dopaminergic deficits in mice (Slikker et al., 1988; Stone et al., 1987a). Moreover, studies have revealed that MDMA administered directly to the brain fails to recapitulate the neurotoxicity seen following systemic administration (Escobedo et al., 2005; Esteban et al., 2001). A number of studies have subsequently investigated the effect of perturbations in MDMA metabolism on MDMA-induced neurotoxicity (Antolino-Lobo et al., 2010; Goni-Allo et al., 2008a; Mueller et al., 2011). The present study was designed to further elaborate the role of COMT in MDMA-induced neurotoxicity.

COMT catalyzes a reaction that yields methoxy metabolites from the catechol substrates N-Me-α-MeDA and α-methyldopamine (α-MeDA). These methoxy metabolites are readily excreted, and unlike the catechol precursors, are unable to form neurotoxic glutathione- or N-acetylcysteine-conjugates (Capela et al., 2007; Miller et al., 1996, 1997). As such, COMT likely serves as a detoxication enzyme during the metabolism of MDMA (Antolino-Lobo et al., 2010). This has significant implications with respect to individual susceptibility to MDMA, because the COMT gene exhibits a number of polymorphisms in humans (Tunbridge, 2010). Genotypes that decrease COMT activity appear to have a negative influence on the cognitive effects of MDMA (Cuyas et al., 2011; Schilt et al., 2009). Our own data reveal that COMT deficiency may potentiate serotonergic neurotoxicity (pharmacological model, Fig. 3) or acute MDMA-induced toxicity (genetic model, Fig. 7). A previous study using the peripherally-restricted COMT inhibitor, entacapone, reveals that pretreatment with entacapone prior to a non-neurotoxic dose of MDMA was sufficient to elicit significant MDMA-induced serotonergic deficits in rat (Goni-Allo et al., 2008a).
Ro 41-0960 is capable of crossing the blood brain barrier and, thus, inhibits both brain and hepatic COMT. Interestingly, despite using a neurotoxic dose of MDMA, serotoninergic deficits in this study (40–65% reduction, Fig. 3) were only slightly greater than those seen in the Goni-Allo study (30–40% reduction), indicating that inhibition of CNS COMT activity likely plays a minor role in enhancing MDMA-induced neurotoxicity. In contrast, inhibition of hepatic COMT activity appears to play a major role in potentiation of MDMA-induced neurotoxicity. Inhibition of COMT may potentiate MDMA-induced neurotoxicity by promoting greater formation of neurotoxic thioether metabolites of MDMA, although this would require validation as concentrations of these metabolites were not measured in this study.

Our results are consistent with findings describing species differences in MDMA-induced neurotoxicity. Thus, mice primarily exhibit a dopaminergic neurotoxicity when administered MDMA, which differs from the MDMA-induced serotonergic neurotoxicity observed in rats (de la Torre and Farre, 2004). This difference in neurotoxic profile may be due to an apparent deficiency in O-demethylation in mice, which results in a larger fraction of the administered dose of MDMA reaching tissues as parent compound in this species (Lim et al., 1992). Mice are also less susceptible to the neurotoxic effects of MDMA, with larger MDMA doses required to elicit monoamine deficits (Stone et al., 1987a). Despite this reduced susceptibility, a single dose of 30 mg/kg MDMA produced lethality in the majority of COMT−/− mice. An increase in mortality was not recapitulated in the rat model of COMT deficiency, perhaps due to the residual (≈10%) COMT activity remaining after Ro 41-0960 treatment (Fig. 2). However, 5-HT deficits were greater in MDMA treated rats pretreated with the COMT inhibitor. Measurement of catecholamine deficits was the index of neurotoxicity we employed in these studies. No further markers of neurodegeneration were assayed. However, other indices of MDMA-induced neurotoxicity, including anatomical aberrations and decreases in serotonin reuptake transporter binding sites or tryptophan hydroxylase expression in rats ( Battaglia et al., 1988; O’Hearn et al., 1988; Stone et al., 1987b), or changes in glial fibrillary acidic protein and tyrosine hydroxylase expression in mice (O’Callaghan and Miller, 1994) are typically accompanied by such neurotransmitter deficits. These data suggest that individuals with lower COMT activity are likely more susceptible to MDMA-induced toxicity than individuals with intermediate or high COMT activity.

We also report that COMT deficiency potentiates the acute MDMA-induced hyperthermia in rats (20 mg/kg, sc, Fig. 4) and mice (30 mg/kg, sc, Fig. 7). Rats treated with MDMA displayed a hyperthermic response regardless of COMT status. However, in WT mice, a single dose of MDMA (30 mg/kg) below the neurotoxic threshold elicited only a mild, and delayed hyperthermic response, subsequent to an initial transient, mild hyperthermia, an effect previously observed in C57Bl/6 mice (Bexis and Docherty, 2009; Sanchez et al., 2003). Hyperthermia has long been debated as a necessary component of MDMA-induced neurotoxicity, at least in the rat. When MDMA is administered to rats (20 mg/kg, sc) maintained at an ambient temperature of 10°C, not only is MDMA-induced hyperthermia abolished, but the animals display a hypothermic response (Gordon et al., 1991). Furthermore, when MDMA-induced hyperthermia is inhibited pharmacologically, with haloperidol for example, depletions in 5-HT are attenuated (Colado et al., 1999). In contrast, inhibition of γ-glutamyl transpeptidase with acivicin potentiated the neurotoxicity of MDMA (Bai et al., 2001) under conditions that actually attenuated MDMA induced hyperthermia (Jones et al., 2005). Our studies on the pharmacological inhibition of COMT reveal a statistically significant potentiation of MDMA-induced hyperthermia in rats pretreated with Ro 41-0960 prior to MDMA (Fig. 4). Furthermore, although a single dose of MDMA (30 mg/kg, sc) failed to produce significant hyperthermia or striatal DA depletion in WT C57Bl/6 mice, it caused marked hyperthermia and death in 80% of COMT−/− mice tested (Fig. 7). The finding that COMT−/− mice exhibit a rapid hyperthermic response following a single dose of MDMA (30 mg/kg, sc) whereas WT mice did not implicates COMT as an enzyme important in preventing potentially lethal MDMA-induced hyperthermia.

Interestingly, male COMT−/− mice were more prone to MDMA-induced lethality than their female counterparts. Males have previously been shown to be more sensitive to the acute toxicity of MDMA (Cadet et al., 1994; Fonsart et al., 2008). Male COMT−/− mice monitored for body temperature consistently exhibited a rapid hyperthermia whereas only 60% of the females exhibited this rapid MDMA-induced hyperthermia (Fig. 7). Unlike male COMT−/− mice, plasma NE concentrations were not significantly elevated in female COMT−/− mice treated with MDMA (Fig. 8). However, the difference in absolute concentrations of plasma NE between male and female COMT−/− mice treated with MDMA is small enough to suggest that other factors likely contribute to this gender difference in MDMA-induced lethality. Estrogen may contribute to this gender difference, as ovariectomized rats without estrogen replacement treatment exhibit a downward shift in the thermoneutral zone, which is the temperature range at which heat dissipation mechanisms are triggered (Dacks and Rance, 2010). Furthermore, estradiol treatment of ovariectomized rats reduced tail skin vasodilation (Dacks and Rance, 2010). Female mice were not assessed for stage of estrous cycle in our study, so perhaps female COMT−/− mice exhibiting MDMA-induced hyperthermia were in the estrus phase of their cycle, when elevated estrogen levels may act to reduce heat dissipation.

MDMA-induced hyperthermia is complex, requiring both central and peripheral components. The hypothalamus is an area of the brain crucial in thermoregulation, as lesions in the hypothalamus produce a marked increase in thermogenesis (Romanovsky et al., 2003). Peripheral MDMA administration causes an increase in extracellular DA in the hypothalamus (Benamar et al., 2008), which is one of the many neurotransmit-
Methylenedioxyamphetamine (MDMA) induces a hyperthermic response in rats, which is mediated by adrenergic receptors. NE is a critical component of MDMA-induced hyperthermia, and COMT inhibition also potentiates neurotoxicity. COMT catalyzes the formation of normetanephrine, thereby terminating the biological activity of NE, which can cause lethal hyperthermia. In our studies, plasma concentrations of NE in rats treated with MDMA were reported to be markedly elevated as early as 30 min after MDMA administration (Sprague et al., 2004). In fact, inhibiting α1 and β3 adrenoceptors in mice (Bexis and Docherty, 2009) attenuates MDMA-induced hyperthermia, likely by facilitating NE release and stimulating the formation of normetanephrine, thereby terminating the biological activity of NE (Eisenhofer et al., 1996). COMT−/− animals are incapable of terminating NE signaling by COMT, likely allowing circulating NE concentrations sufficient to cause lethal hyperthermia. Although we did not assay plasma NE concentrations in our studies, plasma concentrations of NE in rats treated with MDMA are reported to be markedly elevated as early as 30 min after MDMA administration (Sprague et al., 2004). In summary, our data support the contention that MDMA-induced neurotoxicity can be manipulated by modulating the metabolism of MDMA. Consequently, inhibition of COMT, an enzyme that catalyzes the formation of normetanephrine, thereby terminating the biological activity of NE, can serve to decrease levels of the reactive intermediate catechol (N-Me-α-MeDA and α-MeDA) metabolites of MDMA, potentiates neurotoxicity. COMT inhibition also potentiates MDMA-induced hyperthermia, likely by facilitating prolonged NE stimulation of adrenergic receptors. These findings have significant implications for humans, because individuals carrying the low activity variant of the COMT protein would likely be at elevated risk of MDMA-induced toxicity.

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