Thioether Metabolites of 3,4-Methylenedioxyamphetamine and 3,4-Methylenedioxymethamphetamine Inhibit Human Serotonin Transporter (hSERT) Function and Simultaneously Stimulate Dopamine Uptake into hSERT-Expressing SK-N-MC Cells

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ABSTRACT
3,4-Methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) are widely abused amphetamine derivatives that target the serotonin system. The serotonergic neurotoxicity of MDA and MDMA seems dependent on their systemic metabolism. 5-(Glutathion-S-yl)-α-methyldopamine [5-(GSyl)-α-MeDA] and 2,5-bis(glutathion-S-yl)-α-methyldopamine [2,5-bis(GSyl)-α-MeDA], metabolites of MDA and MDMA, are also selective serotonergic neurotoxins and produce behavioral and neurochemical changes similar to those seen with MDA and MDMA. We now show that 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA, and to a lesser extent MDA and MDMA, induced a concentration and time-dependent increase in reactive oxygen species (ROS) in both hSERT and human dopamine transporter-transfected cells. Fluoxetine attenuated the increase in ROS generation in hSERT-expressing cells. The results are consistent with the view that the serotonergic neurotoxicity of MDA and MDMA may be mediated by the metabolism-dependent stimulation of DA transport into hSERT-expressing cells and ROS generation by redox active catechol-thioether metabolites and DA.

Moreover, 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA simultaneously stimulated dopamine (DA) transport into the hSERT-expressing cells, an effect attenuated by fluoxetine, indicating that stimulated DA transport was hSERT-dependent. Finally, 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA, and to a lesser extent MDA and MDMA, induced a concentration and time-dependent increase in reactive oxygen species (ROS) in both hSERT and human dopamine transporter-transfected cells. Fluoxetine attenuated the increase in ROS generation in hSERT-expressing cells. The results are consistent with the view that the serotonergic neurotoxicity of MDA and MDMA may be mediated by the metabolism-dependent stimulation of DA transport into hSERT-expressing cells and ROS generation by redox active catechol-thioether metabolites and DA.

3,4-Methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) are ring-substituted amphetamine derivatives with stimulant and hallucinogenic properties (Ricaurte et al., 1985; Commis et al., 1987). The recreational use of these amphetamines, especially MDMA, is prevalent (Johnston et al., 2000) despite warnings of damage to the central nervous system, (McCann et al., 1999; Parrott, 2000). MDA and MDMA are serotonergic neurotoxins (Ricaurte et al., 2000), the long-term consequences of which are manifest as depletions in 5-HT and 5-hydroxyindoleacetic acid concentrations (Commins et al., 1987). In addition, inhibition of function (Fleckenstein et al., 1999) and loss of 5-HT transporters (ERT) (Battaglia et al., 1987; Aguirre et al., 1998) likely contributes to long-term impairments of the serotonergic system, including the degeneration of 5-HT axonal projections and nerve terminals (O’Hearn et al., 1988).

Interestingly, direct injection of MDA and MDMA into the brain fails to reproduce the acute or long-term neurotoxic effects evident after peripheral administration (Molliver et al., 1986; Paris and Cunningham, 1992; Esteban et al., 2001). Moreover, inhibition of cytochrome P450-mediated MDMA metabolism attenuates MDMA-induced neurotoxicity (Gollamudi et al., 1989). Therefore, systemic metabolism of the parent drugs likely plays an important role in the development of neurotoxicity (Esteban et al., 2001; for review, see Monks and Jones, 2002; O’Shea et al., 2002). Esteban et al. (2001) provide persuasive evidence that peripheral metabo-

ABBREVIATIONS: MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine (ecstasy); 5-HT, 5-hydroxytryptamine (serotonin); SERT, serotonin transporter; GSH, glutathione; 5-(GSyl)-α-MeDA, 5-(glutathion-S-yl)-α-methyldopamine; 2,5-bis(GSyl)-α-MeDA, 2,5-bis(glutathion-S-yl)-α-methyldopamine; DA, dopamine; MAO, monoamine oxidase; ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; hSERT, human serotonin transporter; hDAT, human dopamine transporter; DAT, dopamine transporter; KR, Krebs-Ringer; DCF-DA, dichlorofluorescein diacetate; DCF, dichlorofluorescein.
lism of MDMA is required for neurotoxicity. Sufficient amounts of MDMA were perfused into the hippocampus to achieve the range of concentrations observed after peripheral administration of neurotoxic doses of MDMA. After 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 MDMA metabolites, including α-MeDA, fail to elicit 5-HT neurotoxicity (Zhao et al., 1992; Miller et al., 1997). α-MeDA undergoes rapid oxidation to α-quinones, which are readily scavenged by glutathione (GSH) (Hiramatsu et al., 1990; Patel et al., 1991) to yield 5-glutathionylα-MeDA [5-(GSyl)-α-MeDA] and 2,5-bis(glutathionyl-S-yl)-α-MeDA [2,5-bis(GSyl)-α-MeDA] (Miller et al., 1995; Fig. 1). These catechol-thioethers retain the electrophilic and redox properties of the parent catechols and produce neurotoxic responses similar to MDMA and MDA (Miller et al., 1995, 1997; Bai et al., 1999), suggesting that such metabolites contribute to the neurotoxicity of MDA and MDMA. Furthermore, γ-glutamyl transpeptidase is enriched in blood brain-barrier endothelial cells and catalyzes the first step in GSH and S-conjugate metabolism. The finding that the inhibition of γ-glutamyl transpeptidase potentiates both the brain uptake (Miller et al., 1996) and the neurotoxicity of systemically administered MDA and MDMA (Bai et al., 2001), suggests that the neurotoxicity is mediated in part by metabolites that are substrates for this enzyme.

Although the importance of the SERT in MDA- and MDMA-induced neurotoxicity is well established, the precise function of the transporter remains elusive. Fluoxetine and citalopram, SERT inhibitors, protect against MDMA-induced neurotoxicity (Aguirre et al., 1998; Liebthi et al., 2000; Sanchez et al., 2001), and MDMA-induced depletions of 5-HT are absent in SERT-deficient mice (Bengel et al., 1998), supporting the involvement of a functional SERT in MDMA-induced neurotoxicity. MDMA-induced serotonergic neurotoxicity also seems to be coupled to increases in dopamine (DA) release (Bankson and Cunningham, 2001). MDMA stimulates the release of DA (Gudelsky and Nash, 1996; Koch and Galloway, 1997), and functional SERTs are capable of transporting DA into 5-HT cells (Schmidt and Lovenberg 1985; Faraj et al., 1994). The subsequent monoamine oxidase (MAO)-B-mediated oxidation of DA within the 5-HT nerve terminal may contribute to MDMA-induced generation of reactive oxygen species (ROS; Sprague and Nichols, 1995; Sprague et al., 1998). Indeed, the generation of ROS plays an important role in MDMA-induced neurotoxicity (Colado et al., 1997). Consequently, antioxidants attenuate MDMA-induced neurotoxicity (Gudelsky, 1996; Shankaran et al., 2001), and overexpression of superoxide dismutase protects against the effects of MDMA (Cadet et al., 1995). Lipid peroxidation and protein nitration, morphological markers of ROS-induced damage, are also apparent after MDMA administration (Sprague and Nichols, 1995). The source of ROS generation induced by MDMA remains debatable, although two pathways seem possible: 1) metabolism of MDMA to reduct active metabolites (Miller et al., 1995, 1996; Bai et al., 2001; Esteban et al., 2001, and 2) oxidation of DA (Sprague et al., 1998; Shankaran et al., 1999).

We herein demonstrate that thioether metabolites of α-MeDA inhibit 5-HT transport into hSERT-transfected SK-N-MC cells more effectively than either MDA or MDMA and that they concomitantly stimulate the uptake of DA into hSERT-expressing cells. Moreover, thioether metabolites of α-MeDA stimulate ROS generation in hSERT-transfected SK-N-MC cells in a hSERT-dependent manner. The combination of the oxidation of DA and of the thioether metabolites of α-MeDA likely contributes to the generation of ROS within 5-HT neurons and to MDA- and MDMA-induced neurotoxicity.

**Materials and Methods**

**Chemicals.** MDA and MDMA were supplied by the Research Technology Branch (National Institute on Drug Abuse, Rockville, MD). ['H]5-HT and ['H]DA were obtained from American Radiolabeled Chemicals (St. Louis, MO). Fluoxetine, nomifensine, 2,7-di-chlorofluorescein diacetate, mushroom tyrosinase, and GSH were purchased from Sigma-Aldrich (St. Louis, MO). 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA were synthesized and purified as described previously (Miller et al., 1995). Briefly, 5-(GSyl)-α-MeDA was prepared by reacting α-MeDA (Merck Research Labs, Rahway, NJ) (2 mM), GSH (10 mM), and mushroom tyrosinase (100 U/ml) in 100 ml of sodium phosphate buffer (50 mM, pH 7.4) The product was purified by HPLC (HPLC-6A; Shimadzu, Kyoto, Japan) using an Ultrasphere ODS-5 reverse-phase semipreparative column (Beckman Coulter, Fullerton, CA). Fractions were collected at λ = 280 nm/
retention time of 12 min. Collected fractions were combined, concentrated by rotary evaporation, frozen over dry ice/acetone, and lyophilized to dryness. The resulting powder was reanalyzed by HPLC and coulometric electrode array detection. 2,5-bis(GSyl)-α-MeDA was synthesized by dissolving 100 mg of 5-(GSyl)-α-MeDA in 100 ml of 10% formic acid. Sodium periodate (50 mg) was added to the solution to promote quinone formation before saturating the reaction with GSH. The resulting mixture was concentrated by rotary evaporation, frozen over dry ice/acetone, and lyophilized to dryness. The product was purified by HPLC, and the major UV-absorbing product was eluted with water. Fractions were collected at λ = 280/nm retention time of 11 min, rotary evaporated, and lyophilized to dryness. Reanalysis of the product by HPLC with UV and coulometric electrode array detection produced a single peak. All other reagents were purchased from commercial sources.

**Transient Transfection of hsERT and hDAT in SK-N-MC Cells.** SK-N-MC cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Eagle’s minimal essential medium (American Type Culture Collection) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The parental cDNAs, hsERT, and hDAT, inserted into pcDNA 3.1 (Invitrogen, Carlsbad, CA), were used for transient expression of the transporter proteins. Transfections were accomplished using the Lipofectamine reagent (Invitrogen) following the protocol supplied by the manufacturer. Briefly, 24 h before transfection, cells were seeded in 24-well plates (~5 × 10^5 cells/well). Opti-MEM I reduced serum medium (Invitrogen) containing the cDNA was combined with the Lipofectamine reagent and incubated at room temperature for 30 min. Cells were washed with serum-free medium (Opti-MEM), and the cDNA-liposomal solution was added to each well. Cells were incubated with the cDNA complexes at 37°C, 5% CO₂, for 5 h. Growth medium supplemented with 10% fetal bovine serum was added to each well, and the cells were incubated for 24 h before being replaced with Eagle’s minimal essential medium. Expression of the transporter proteins was confirmed by Western analysis using polyclonal antibodies against the hDAT and hSERT (Research Diagnostics, Flanders, NJ) (Fig. 2) and cellular uptake assays. Transfected cells were used for experiments 48 to 72 h post-transfection.

**Cellular Neurotransmitter Uptake Experiments.** Cellular uptake of [³H]5-HT and [³H]5-HT into hDAT- and hSERT-transfected SK-N-MC cells was measured 48 h post-transfection, as described previously (Mortensen et al., 2001). Several uptake experiments were conducted, and details for each experiment can be found in the figure legends. Briefly, cells were washed with Krebs-Ringer (KR) buffer and exposed to MDA, MDMA, 5-(GSyl)-α-MeDA, or 2,5-bis(GSyl)-α-MeDA for 30 min, and intracellular [³H]5-HT accumulation was determined. Assuming Michaelis-Menten kinetics, substrate Km and inhibitor Ki values were determined by nonlinear least-squares curve fit (GraphPad Prism; GraphPad Software Inc., San Diego, CA; Adkins et al., 2001). Experiments were carried out in triplicate, and Ki values are presented as the mean (n = 4) ± the standard error of four independent transfections.

**Evaluation of ROS Generation.** 2′,7′-Dichlorofluorescein diacetate (DCF-DA) is oxidized to the fluorescent 2′,7′-dichlorofluorescein by cellular oxidants and is used as a marker for intracellular generation of ROS, in particular hydrogen peroxide and the hydroxyl radical. Intracellular ROS generation was monitored as described previously (Jones et al., 2000) with modifications. Transfected SK-N-MC cells were loaded with DCF-DA (final concentration of 10 μM) in KR buffer for 15 min in the dark. Cells were washed with KR buffer and exposed to MDA, MDMA, 5-(GSyl)-α-MeDA, or 2,5-bis(GSyl)-α-MeDA in the presence or absence of the transporter blockers, nomifensine (DAT), and fluoxetine (SERT). Assays were terminated by washing cells with KR buffer and ROS generation was monitored at 475 nm (excitation) and 525 nm (emission) using a FL600 microplate fluorescence reader (Bio-Tek Instruments, Winoski, VT). Changes in fluorescence are expressed as percentage of control, and represent DCF fluorescence in treated samples minus the background fluorescence of the sample (cells treated with the Krebs-Ringer vehicle alone, minus drugs and metabolites, and without DCF-DA). Vehicle controls represent DCF fluorescence in cells to which only DCF-DA was added. This “vehicle control” fluorescence presumably reflects the inherent endogenous level of ROS production in these cells.

**Statistics.** Data are expressed as the mean ± S.E.M. A one-way analysis of variance was performed on the data and multiple pair-
wise comparisons were made using Student-Newman-Keuls tests. Differences within and between treatment groups were considered significant at \( p < 0.05 \).

**Results**

**Inhibition of 5-HT Transport into hSERT-Transfected Cells.** SK-N-MC cells transiently transfected with either pcDNA, hSERT, or hDAT cDNA were used to examine the effect of MDA, MDMA, 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA on the cellular uptake of 5-HT. Confirmation of protein expression was demonstrated by Western analysis and by quantifying the cellular uptake of \([^{3}H]5\text{-HT}\) and \([^{3}H]5\text{DA}\) into hDAT- and hSERT-transfected cells (Fig. 2). Fluoxetine and nomifensine significantly inhibited \([^{3}H]5\text{-HT}\) and \([^{3}H]5\text{DA}\) uptake into hSERT- and hDAT-transfected cells, respectively. The cellular uptake of 5-HT into untreated hSERT-expressing cells was rapid and accumulation continued for 8 h, before slowly returning to baseline levels by 48 h (Fig. 3). MDA, MDMA, 5-GSyl-α-MeDA, and 2,5-bis(GSyl)-α-MeDA maximally inhibited 5-HT uptake between 2 and 4 h after drug treatment (Fig. 3). After 4 h of exposure, MDA and MDMA inhibited 5-HT uptake by approximately 30% (Fig. 4). 5-GSyl-α-MeDA and 2,5-bis(GSyl)-α-MeDA were more potent inhibitors of 5-HT transport than either MDA or MDMA, inhibiting uptake by approximately 60 and 70%, respectively. Nomifensine, a selective DAT inhibitor, had no effect on 5-HT uptake into hSERT-transfected cells (Fig. 4) and did not influence drug or metabolite mediated inhibition of 5-HT uptake. In contrast, 100 μM fluoxetine inhibited 5-HT uptake into hSERT-transfected cells to a similar extent as 5-GSyl-α-MeDA and 2,5-bis(GSyl)-α-MeDA. None of the compounds tested had any effect on mock- (pcDNA) or hDAT-transfected cells (data not shown).

Kinetic analysis of the inhibition of 5-HT uptake was performed on hSERT-transfected cells. \( K_m \) and \( V_{max} \) values for specific \([^{3}H]5\text{-HT}\) uptake were determined by saturation transport analysis using increasing concentrations of \([^{3}H]5\text{-HT}\) (Fig. 5A). \( K_i \) values for each of the compounds were determined by measuring uptake of a single concentration of \([^{3}H]5\text{-HT}\) (20 nM) and various concentrations of MDA, MDMA, 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA. Consistent with the single concentration and time course results, 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA were more potent at inhibiting \([^{3}H]5\text{-HT}\) uptake (Fig. 5B).

**5-(GSyl)-α-MeDA and 2,5-Bis(GSyl)-α-MeDA-Induced ROS Generation.** MDA, MDMA, 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA induce rapid ROS generation in hSERT- and hDAT-transfected SK-N-MC cells (Fig. 6). The rate of ROS generation declines rapidly after the initial burst and returns to baseline levels, presumably due to either metabolism of the drugs and/or exhaustion of reducing equivalents required to support redox cycling. 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA were more efficient inducers of ROS generation, in comparison with MDA and MDMA, in both hSERT- and hDAT-transfected cells (Fig. 7). Although ROS generation was slightly greater in hSERT-transfected cells, the kinetics of ROS generation were similar in both cell types. None of the compounds tested had any effect on ROS generation in mock-transfected cells (data not shown), indicating the requirement for hSERT or hDAT for ROS generation. Interestingly, pretreatment with nomifensine had no
effect on ROS generation in hDAT-transfected cells (Fig. 8B), despite the fact that nomifensine inhibited DA uptake into hDAT-transfected cells (Fig. 2), indicating that ROS generation, although hDAT-dependent, is insensitive to nomifensine. In contrast, fluoxetine significantly inhibited ROS generation in hSERT-transfected cells (Fig. 8A), indicating that MDA, MDMA, 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA induced ROS generation in hSERT-expressing cells requires a functional SERT.

5-(GSyl)-α-MeDA- and 2,5-Bis(GSyl)-α-MeDA-Induced Stimulation of DA Uptake into hSERT-Transfected Cells. DA may contribute to the serotonergic neurotoxicity of MDMA via its ability to generate ROS and reactive quinones (Sprague et al., 1998; Shankaran et al., 1999). MDA, MDMA, 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA all stimulated the cellular uptake of [3H]DA into hSERT-transfected cells (Fig. 9). 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA increased DA uptake ~6- to 7-fold over control levels, whereas MDA and MDMA increased DA uptake by only ~3.5-fold. The maximum stimulation of DA uptake occurred between 4 and 8 h (Fig. 9). Thus, the interaction of the thioether metabolites with the hSERT 1) inhibits the uptake of 5-HT and 2) stimulates the simultaneous uptake of DA into hSERT-expressing cells. Moreover, fluoxetine inhibited the uptake of DA (Fig. 10), supporting the contention that DA is transported into hSERT-transfected cells via the hSERT. Subsequent MAO-mediated DA oxidation and metabolism may then contribute to the generation of ROS and to the serotonergic neurotoxicity of MDA and MDMA.

Discussion

We have demonstrated that 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA stimulate the transport of DA into hSERT-expressing SK-N-MC cells (Fig. 9), while simultaneously inhibiting 5-HT uptake (Figs. 3–5). The stimulation of DA uptake into “serotonergic” cells is a significant finding, because although a relationship between DA- and MDMA-induced serotonergic neurotoxicity has long been appreciated, the mechanisms underlying this relationship are unknown. Consistent with the hypothesis that systemic metabolism is required for MDA and MDMA neurotoxicity, 5-(GSyl)-α-MeDA- and 2,5-bis(GSyl)-α-MeDA-stimulated up-
take of DA into hSERT-expressing SK-N-MC cells was greater than that caused by the parent amphetamines. Fluoxetine attenuated the stimulation of DA uptake (Fig. 10), indicating that DA entered the cell via the hSERT. Indeed, functional SERT proteins are capable of transporting DA into 5-HT cells (Schmidt and Lovenberg, 1985; Faraj et al., 1994). Although the mechanism by which MDMA/MDA and the metabolites stimulate DA uptake by the hSERT is not known, Saldana and Barker (2004) have recently reported that elevated temperature alters SERT-mediated 5-HT and DA transport, such that the relative selectivity of the SERT for DA increases significantly at the higher temperature. Thus, drug and/or metabolite-induced alterations in hSERT structure may similarly alter the relative preference of the transporter for DA and 5-HT.

The importance of the SERT in contributing to MDA- and MDMA-induced neurotoxicity is firmly established (Shankaran et al., 1999, 2001) and neurotoxicity (Aguirre et al., 1998; Sanchez et al., 2001) and citalopram attenuates the effects of MDMA in humans (Liechti et al., 2000), suggesting that the SERT participates in MDMA-mediated cellular oxidant generation and neurotoxicity. Our data are also consistent with an important role for the SERT in MDA and MDMA induced neurotoxicity. For instance, 5-(GSyl)-H9251-MeDA and 2,5-bis(GSyl)-H9251-MeDA significantly inhibited [3H]5-HT transport into hSERT-transfected cells (Fig. 3) and to a greater extent than that produced by either MDA or MDMA. Indeed, the Ki for the thioether metabolites were substantially less than that for either MDA or MDMA (Fig. 5). The interaction between MDMA/MDA and the SERT in vivo may therefore involve both direct (MDA) and indirect (metabolite) effects. Fluoxetine potentiates and nomifensine significantly inhibited [3H]5-HT transport into hSERT-transfected cells (Fig. 3) and to a greater extent than that produced by either MDA or MDMA. Indeed, the Ki for the SERT and nomifensine-treated groups are significantly different at p < 0.05.

Fig. 7. Concentration-dependent ROS generation by MDA, MDMA, 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA in hSERT- and hDAT-transfected SK-N-MC cells. ROS generation was determined in hSERT- (A) and hDAT- (B)-transfected cells using increasing concentrations (10, 50, 100, and 200 μM) of the compounds and by measuring increases in DCF fluorescence 4 h after treatment. Control (●), MDA (△), MDMA (▼), 5-(GSyl)-α-MeDA (○), or 2,5-bis(GSyl)-α-MeDA (▲). Data are expressed as the mean (n = 4 ± S.E.M., and differences between 1) control and treatment groups (**) and 2) MDMA/MDA and metabolite groups (†) are considered significant at p < 0.05.

Fig. 8. Fluoxetine attenuates MDA-, MDMA-, 5-(GSyl)-α-MeDA-, and 2,5-bis(GSyl)-α-MeDA-induced ROS generation in hSERT-expressing cells. ROS generation was determined in hSERT- (A) and hDAT (B)-transfected cells after a 4-h incubation with the compounds by measuring changes in DCF fluorescence. Vehicle control (black columns), MDA (100 μM, open columns), MDMA (100 μM, gray columns), 5-(GSyl)-α-MeDA (100 μM, hatched columns), or 2,5-bis(GSyl)-α-MeDA (100 μM, gradient columns) data are presented as the percentage of increase in DA uptake. Groups of cells were incubated with fluoxetine (100 μM) or nomifensine (50 μM) 20 min before treatment. Data are expressed as the mean (n = 4 ± S.E.M., and differences between 1) vehicle control and treatment groups (**) and 2) MDA/MDA and metabolite groups (†) are considered significant at p < 0.05. §, significantly different from untreated and nomifensine-treated groups at p < 0.05.
considered significant at treatment groups (§) and 2) metabolite groups and MDA/MDMA (\(\text{H11569}^\dagger\)) are

\[ n = 8, 16, 24, \text{and 48 h} \]

by liquid scintillation spectroscopy. Data are ex-

MDMA- (100 \(\mu\text{M}\), \(\equiv\)) and 5-(GSyl)-\(\alpha\)-MeDA- (100 \(\mu\text{M}\), \(\Delta\))-treated SK-N-MC cells at various times (0.5, 1, 2, 4, 8, 16, 24, and 48 h) by liquid scintillation spectroscopy. Data are ex-

MDMA- (100 \(\mu\text{M}\), \(\equiv\)), 5-(GSyl)-\(\alpha\)-MeDA- (100 \(\mu\text{M}\), \(\Delta\)), or 2,5-bis(GSyl)-\(\alpha\)-MeDA (100 \(\mu\text{M}\), ○) treated SK-N-MC cells at various times (0.5, 1, 2, 4, 8, 16, 24, and 48 h) by liquid scintillation spectroscopy. Data are ex-

MDMA- (100 \(\mu\text{M}\), \(\equiv\)), 5-(GSyl)-\(\alpha\)-MeDA- (100 \(\mu\text{M}\), \(\Delta\)), or 2,5-bis(GSyl)-\(\alpha\)-MeDA (100 \(\mu\text{M}\), ○) treated SK-N-MC cells at various times (0.5, 1, 2, 4, 8, 16, 24, and 48 h) by liquid scintillation spectroscopy. Data are ex-

MDMA- (100 \(\mu\text{M}\), \(\equiv\)), 5-(GSyl)-\(\alpha\)-MeDA- (100 \(\mu\text{M}\), \(\Delta\)), or 2,5-bis(GSyl)-\(\alpha\)-MeDA (100 \(\mu\text{M}\), ○) treated SK-N-MC cells at various times (0.5, 1, 2, 4, 8, 16, 24, and 48 h) by liquid scintillation spectroscopy. Data are ex-

interaction with hSERT is unknown. However, the electrophilic nature of 5-(GSyl)-\(\alpha\)-MeDA and 2,5-bis(GSyl)-\(\alpha\)-MeDA provides a means by which the metabolites may interact with nucleo-

philic sites in the SERT, such as the multiple cysteine residues found in the extracellular domain (Chen et al., 1998), to modify SERT function.

Inhibition of SERT function has been attributed to MDMA-

induced ROS generation (Sprague and Nichols, 1995; Falk et al., 2002); whereas, conversely, an increase in ROS generation may be a consequence of MDMA-induced SERT inhibi-

tion (Shankaran et al., 1999, 2001). In accord with previous reports (Shankaran et al., 1999, 2001), our data demonstrate that MDA and MDMA induced significant increases in ROS generation in hSERT-expressing cells (Fig. 6). However, 5-(GSyl)-\(\alpha\)-MeDA and 2,5-bis(GSyl)-\(\alpha\)-MeDA were more ef-

te ROS generators than either MDA or MDMA, supporting the view that systemic metabolism contributes to the serotonergic neurotoxicity of the parent drugs. Moreover, fluoxetine attenuated the increase in ROS generation in hSERT-expressing cells (Fig. 8), indicating that MDA, MDMA, 5-(GSyl)-\(\alpha\)-MeDA, and 2,5-bis(GSyl)-\(\alpha\)-MeDA induced ROS generation in serotonergic cells is SERT-depen-

dent.

DA oxidation and the subsequent generation of ROS have been implicated in MDMA-induced serotonergic neurotoxicity (Sprague and Nichols, 1995; Aguirre et al., 1998; Sprague et al., 1998; Shankaran et al., 1999; Bankson and Cunningham, 2001), although the mechanism by which MDMA pro-
duces these effects is not known. Both DA (Simantov and Tauber, 1997) and dopa (Schmidt et al., 1991; Aguirre et al., 1998) potentiate the neurotoxicity of MDMA. Fur-

the, inhibition of MAO-B, which metabolizes DA within the serotonin cell, protects against serotonergic cell damage (Sprague and Nichols, 1995; Falk et al., 2002) presumably by preventing the oxidation of DA and the subsequent ROS generation. We have now shown that not only can the hSERT modestly transport dopamine into hSERT-expressing cells but also that such hSERT-mediated DA transport is greatly stimulated by 5-(GSyl)-\(\alpha\)-MeDA and 2,5-bis(GSyl)-\(\alpha\)-MeDA, and to a lesser extent by MDMA and MDA (Figs. 9 and 10). These data may provide the crucial link between MDMA- and MDA-mediated serotonergic neurotoxicity and the long rec-

ognized requirement for DA for these effects. However, we note that the maximum stimulation of DA uptake by 5-(GSyl)-\(\alpha\)-MeDA and 2,5-bis(GSyl)-\(\alpha\)-MeDA occurred sub-

sequent to maximum ROS generation. This is likely a conse-

quence of the rapid depletion of reducing equivalents, re-

quired to support redox cycling, which typically occurs in cul-

tured cells. Nonetheless, the data do reveal a second wave of ROS generation in hDAT-transfected cells (Fig. 6) concomi-
tant with maximum drug-induced DA uptake (Fig. 9). DA uptake and oxidation within serotonergic neurons may thus contribute to sustained ROS generation consequent to that initially catalyzed by MDA, MDMA, and their metabolites. MDMA-induced ROS generation therefore probably involves multiple mechanisms, including the oxidation and redox cycling of 5-(GSyl)-\(\alpha\)-MeDA and 2,5-bis(GSyl)-\(\alpha\)-MeDA (Hiramatsu et al., 1990; Miller et al., 1996; Bai et al., 1999) and the oxidation of DA within serotonergic nerve terminals (Sprague and Nichols, 1995; Aguirre et al., 1998; Sprague et al., 1998) after drug and/or metabolite stimulated DA uptake.

Interestingly, neither MDMA/MDA- nor 5-(GSyl)-\(\alpha\)-MeDA-

and 2,5-bis(GSyl)-\(\alpha\)-MeDA-stimulated ROS generation in hDAT-expressing cells was inhibited by nomifensine (Fig. 8B). Moreover, nomifensine failed to inhibit MDMA/MDA- or 5-(GSyl)-\(\alpha\)-MeDA- and 2,5-bis(GSyl)-\(\alpha\)-MeDA-stimulated DA uptake into hDAT-expressing cells (data not shown). We speculate that exposure of the hDAT to MDA/MDA/metab-
olite causes physical alterations in the transporter that render it insensitive to nonfenisine. Consistent with this view, the effects of MDMA on DA uptake in striatal synaptosomes are insensitive to the DAT ligand 2β-carboxymethoxy-3β-(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate (WIN35428) (Hansen et al., 2002), further suggesting that MDMA interacts with the DAT in a novel manner insensitive to modulation by classical inhibitors of DAT function.

In summary, 5-(GSyl)-alpha-MeDA and 2,5-bis(GSyl)-alpha-MeDA stimulate the transport of DA into hSERT-expressing SK-N-MC cells, while simultaneously inhibiting 5-HT uptake. The findings provide a possible mechanism underlying the interaction between the dopaminergic and serotonergic neurotransmitter systems during MDA- and MDMA-mediated neurotoxicity. In essence, the systemic metabolism of the parent amphetamines likely contributes to the serotonergic neurotoxicity by compromising SERT function and stimulating the uptake of DA into 5-HT cells, where it subsequently undergoes oxidation. Finally, the demonstration that 5-(GSyl)-alpha-MeDA and 2,5-bis(GSyl)-alpha-MeDA can inhibit hSERT-mediated 5-HT uptake not only illustrates the pharmacological properties of these metabolites but is consistent with their in vivo properties.

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