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THIOETHER METABOLITES OF MDA AND MDMA INHIBIT hSERT FUNCTION AND SIMULTANEOUSLY STIMULATE DOPAMINE UPTAKE INTO hSERT-EXPRESSING SK-N-MC CELLS

By

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Running title: Metabolites of MDA and MDMA compromise SERT function

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Abbreviations: DA, dopamine; DAT, dopamine transporter; 5-(GSyl)-α-MeDA, 5-(Glutathion-*S*-yl)-α-methyldopamine; 2,5-*bis*(GSyl)-α-MeDA, 2,5-*bis*(glutathion-*S*-yl)-α-methyldopamine; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-Hydroxytryptamine (serotonin); MDA, 3,4-Methylenedioxyamphetamine; MDMA, 3,4-Methylenedioxymethamphetamine (ecstasy); ROS, reactive oxygen species; SERT, serotonin transporter.

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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 appears 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 neurotoxicants, 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 are more potent than MDA and MDMA (Ki = 69, 50, 107, and 102 μM, respectively) at inhibiting serotonin (5-HT) transport into SK-N-MC cells transiently transfected with the human serotonin transporter (hSERT). 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 (hDAT)-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.

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3,4-Methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA, ecstasy) are ring-substituted amphetamine derivatives with stimulant and hallucinogenic properties (Ricaurte *et al.,* 1985; Commins *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 neurotoxicants (Ricaurte *et al*., 2000), the long-term consequences of which are manifest as depletions in 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations (Commins *et al.,* 1987). In addition, inhibition of function (Fleckenstein *et al.,* 1999) and loss of of 5-HT transporters (SERT) (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 following peripheral administration (Molliver *et al.,* 1986; Paris and Cunningham, 1992, Esteban *et al.,* 2001). Moreover, inhibition of cytochrome P-450-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; Monks and Jones [review], 2002; O'Shea *et al.,* 2002). Esteban *et al*., (2001) provide persuasive evidence that peripheral metabolism of MDMA is required for neurotoxicity. Sufficient amounts of MDMA were perfused into the hippocampus to achieve 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-

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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 *o*-quinones, which are readily scavenged by glutathione (GSH) (Hiramatsu *et al.,* 1990; Patel *et al.,* 1991) to yield 5-(glutathion-*S*-yl)-α-MeDA (5-(GSyl)-α-MeDA) and 2,5-*bis*(glutathion-*S*-yl)-α-MeDA (2,5-*bis*(GSyl)-α-MeDA) (Miller *et al.,* 1995; Figure 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 (γ-GT) 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-GT 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; Liechti *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 appears to be coupled to increases in dopamine (DA) release (Bankson and Cunningham, 2001). MDMA stimulates the release of DA (Guldelsky and Nash, 1996; Koch and Galloway, 1997) and functional SERT's are capable of transporting DA into 5-HT cells (Faraj *et al.,* 1994; Schmidt and Lovenberg 1985). 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 *et al.*, 1998; Sprague and Nichols; 1995). 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 over-expression 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 following MDMA administration (Sprague and Nichols, 1995). The source of ROS generation induced by MDMA remains debatable, although two pathways seem possible, (i) the metabolism of MDMA to redox active metabolites (Miller *et al.*, 1995, 1996; Bai *et al*, 2000; Esteban *et al.*, 2001) and, (ii) the 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.

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METHODS: Chemicals. MDA and MDMA were supplied by the Research Technology Branch, National Institute on Drug Abuse, Rockville, MD. [^{3}H] 5-HT and [^{3}H] DA were obtained from Radiolabeled Chemicals Inc (St. Louis, MO). Fluoxetine, nomifensine, 2'7'-dichlorofluorescein diacetate, mushroom tyrosinase, and GSH were purchased from Sigma (St. Louis, MO). 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA were synthesized and purified as previously described (Miller *et al.,* 1995). Briefly, 5-(GSyl)-α-MeDA was prepared by reacting α-MeDA (Merck Research Laboratories, Rahway, NJ) (2mM), GSH (10mM), and mushroom tyrosinase (100U/ml) in 100 ml sodium phosphate buffer (50mM, pH 7.4). The product was purified by HPLC (Shimadzu, LC-6A) using a Beckman Ultrasphere ODS-5 reverse-phase semi-preparative column. 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 re-analyzed 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 prior to 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/retention time of 11 min, rotary evaporated and lyophilized to dryness. Re-analysis 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 (ATCC; Manassas, VA). Cells were cultured in

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Eagles Minimal Essential medium (ATCC) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The parental cDNA's, hSERT and hDAT, inserted into pCDNA 3.1 (Invitrogen), 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 hrs prior to transfection, cells were seeded in 24 well plates (~5 X 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% CO_2, for 5 hours. Growth medium supplemented with 10% fetal bovine serum was added to each well and the cells were incubated for 24 hrs before being replaced with Eagles Minimal Essential medium. Expression of the transporter proteins was confirmed by western analysis using polyclonal antibodies against the hDAT and hSERT (Research Diagnostics Inc., Flanders, NJ) (Figure 2) and cellular uptake assays. Transfected cells were used for experiments 48-72 hrs post transfection.

Cellular Neurotransmitter Uptake Experiments. Cellular uptake of [^{3}H]-DA and [^{3}H]-5-HT into hDAT and hSERT transfected SK-N-MC cells was measured 48 hours post transfection, as previously described (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 buffer (NaCl, 125 mM; KCl, 5 mM; Hepes, 25 mM, glucose, 6 mM; $NaHCO_3$, 5 mM; $MgSO_4*7H_2O$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2*2H_2O$, 2.4 mM; pH, 7.4). Cells were then incubated in KR buffer containing 20 nM [^{3}H]5-HT, 100 μM pargyline and 100 μM ascorbate plus MDA, MDMA, 5-(GSyl)-α-MeDA, or 2,5-*bis*-(GSyl)-α-MeDA (100 μM).

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Cellular uptake was terminated by washing the cells with Krebs-Ringer buffer and uptake of [^{3}H]5-HT into mock-, hDAT-, and hSERT-transfected cells was determined by liquid scintillation spectroscopy (Beckman LS 5000TD) and cpms normalized per cell number. Specific [^{3}H]5-HT uptake is defined as that fraction of tritium uptake inhibitable by fluoxetine. The cellular uptake of [^{3}H]DA into the hSERT-transfected cells was examined using a procedure similar to that used for [^{3}H]5-HT, and specific uptake defined as that fraction of tritium uptake inhibitable by nomifensine.

The kinetics of 5-(GSyl)-α-MeDA and 2,5-*bis*-(GSyl)-α-MeDA-induced inhibition of 5-HT cellular uptake was examined with saturation and inhibition experiments. Saturation transport analysis was conducted by incubating the cells with increasing concentrations of substrate ([^{3}H]5-HT) for 30 min in Krebs-Ringer buffer containing 100 μM pargyline and 100 μM ascorbate. Non-specific uptake was determined in parallel using fluoxetine (10 μM). Assays were terminated by washing the cells with Krebs-Ringer buffer and intracellular accumulation of [^{3}H]5-HT was determined by liquid scintillation spectroscopy. For inhibition experiments, cells were incubated in Krebs-Ringer buffer containing 20 nM [^{3}H]5-HT and increasing concentrations of MDA, MDMA, 5-(GSyl)-α-MeDA, or 2,5-*bis*-(GSyl)-α-MeDA for 30 min, and intracellular [^{3}H]5-HT accumulation was determined. Assuming Michaelis-Menten kinetics, substrate Km and inhibitor Ki values were determined by non-linear 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 previously described (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 475nm (excitation) and 525nm (emission) using a FL600 microplate fluorescence reader (Bio-tex). Changes in fluorescence are expressed as % control and represent DCF fluorescence in treated samples minus the "background" fluorescence of the sample (cells treated with the Kreb's 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 ± SEM. A one-way analysis of variance (ANOVA) was performed on the data and multiple pairwise comparisons were made using Student Newman-Kuels tests. Differences within and between treatment groups were considered significant at $p < 0.05$

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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-HT and [^{3}H]DA into hDAT and hSERT-transfected cells (Figure 2). Fluoxetine and nomifensine significantly inhibited [^{3}H]5-HT and [^{3}H]DA uptake into hDAT and hSERT-transfected cells, respectively. The cellular uptake of 5-HT into untreated hSERT-expressing cells was rapid and accumulation continued for 8 hours, before slowly returning to baseline levels by 48 hrs (Figure 3). MDA, MDMA, 5-GSyl-α-MeDA, and 2,5-bis-GSyl-α-MeDA maximally inhibited 5-HT uptake between 2-4 hrs following drug treatment (Figure 3). Following 4 hours of exposure, MDA and MDMA inhibited 5-HT uptake by approximately 30% (Figure 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 (Figure 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 SERT transfected cells. K_m and V_{max} values for specific [^{3}H]5-HT uptake were determined by saturation transport analysis using increasing concentrations of [^{3}H]5-HT (Figure 5A). K_i values for each of the compounds were determined by measuring uptake of a single concentration of [^{3}H]5-HT (20 nM) and various concentrations of MDA, MDMA, 5-(G*S*yl)-α-MeDA, and 2,5-*bis*(G*S*yl)-α-

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MeDA. Consistent with the single concentration and time course results, 5-(G*S*yl)-α-MeDA, and 2,5-*bis*(G*S*yl)-α-MeDA were more potent at inhibiting [^{3}H]5-HT uptake (K_i = 69 and 49 μM, respectively) than either MDA or MDMA (Ki = 107 and 102 μM, respectively) (Figure 5B).

5-(GSyl)-α-MeDA, and 2,5-*bis*(GSyl)-α-MeDA-induced ROS generation. MDA, MDMA, 5-(G*S*yl)-α-MeDA, and 2,5-*bis*(G*S*yl)-α-MeDA induce rapid ROS generation in hSERT- and hDAT-transfected SK-N-MC cells (Figure 6). The rate of ROS generation declines rapidly after the initial burst, and quickly returns to baseline levels, presumably due to either metabolism of the drugs and/or exhaustion of reducing equivalents required to support redox cycling. 5-(G*S*yl)-α-MeDA, and 2,5-*bis(*G*S*yl)-α-MeDA were more efficient inducers of ROS generation, in comparison to MDA and MDMA, in both hSERT and hDAT transfected cells (Figure 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 (Figure 8B), despite the fact that nomifensine inhibited DA uptake into hDAT-transfected cells (Figure 2), indicating that ROS generation, although hDAT-dependent, is insensitive to nomifensine. In contrast, fluoxetine significantly inhibited ROS generation in hSERT-transfected cells (Figure 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. 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 [^{3}H]DA into hSERT-transfected cells (Figure 9). 5-(GSyl)-α-MeDA, and 2,5-bis(GSyl)-α-MeDA increased DA uptake ~6-7-fold over control levels, whereas MDA and MDMA increased DA uptake by only ~3.5-fold. The maximum stimulation of DA uptake occurred between 4-8 hrs (Figure 9). Thus, the interaction of the thioether metabolites with the hSERT; i) inhibits the uptake of 5-HT and, ii) stimulates the simultaneous uptake of DA into hSERT-expressing cells. Moreover, fluoxetine inhibited the uptake of DA (Figure 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 (Figure 9), whilst simultaneously inhibiting 5-HT uptake (Figures 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 uptake 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 (Figure 10), indicating that DA entered the cell *via* the hSERT. Indeed, functional SERT proteins are capable of transporting DA into 5-HT cells (Faraj *et al.,* 1994; Schmidt and Lovenberg, 1985). 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 (Liechti *et al.,* 2000; Shankaran *et al.,* 1999), although the precise role of the SERT remains unclear. MDMA-induced depletion of 5-HT and hyperactivity are both absent in SERT-deficient mice (Bengel *et al.,* 1998) suggesting that a functional SERT is essential for MDMA-induced neurotoxicity. The SERT inhibitor fluoxetine protects against MDMA-induced ROS generation (Shankaran *et al.,* 1999, 2001) and neurotoxicity (Aguirre *et al.,* 1998, Sanchez

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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)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA significantly inhibited [^{3}H]5-HT transport into hSERT transfected cells (Figure 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 (Figure 5). The interaction between MDMA/MDA and the SERT *in vivo* may therefore involve both direct (MDMA) and indirect (metabolite) effects. Fluoxetine potentiated MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA inhibition of 5-HT uptake, suggesting the compounds act in synergy with fluoxetine, although the precise location and nature of this interaction with hSERT is unknown. However, the electrophilic nature of 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA provides a means by which the metabolites may interact with nucleophilic 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 inhibition (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 (Figure 6). However, 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA were more effective 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

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the increase in ROS generation in hSERT-expressing cells (Figure 8), indicating that MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA induced ROS generation in "serotonergic" cells is SERT dependent.

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 produces these effects is not known. Both DA (Simantov and Tauber, 1997) and L-dihydroxyphenylalanine (L-dopa) (Schmidt *et al.*, 1991; Aguirre *et al.*, 1998) potentiate the neurotoxicity of MDMA. Furthermore, inhibition of monoamine oxidase B (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 that such hSERT-mediated DA transport is greatly stimulated by 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA, and to a lesser extent by MDMA and MDA (Figures 9 and 10). These data may provide the crucial link between MDMA and MDA-mediated serotonergic neurotoxicity, and the long recognized requirement for DA for these effects. However, we note that the maximum stimulation of DA uptake by 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA occurred subsequent to maximum ROS generation. This is likely a consequence of the rapid depletion of reducing equivalents, required to support redox cycling, which typically occurs in cultured cells. Nonetheless, the data do reveal a second wave of ROS generation in hDAT transfected cells (Figure 6) concomitant with maximum drug-induced DA uptake (Figure 9). DA uptake and oxidation within serotonergic neurons may thus contribute to sustained ROS generation subsequent 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)-α-MeDA and 2,5-*bis*(GSyl)-α-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; Sprague *et al.,* 1998; Aguirre *et al.,* 1998) following drug and/or metabolite stimulated DA uptake.

Interestingly, neither MDMA/MDA or 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA stimulated ROS generation in hDAT expressing cells was inhibited by nomifensine (Figure 8B). Moreover, nomifensine failed to inhibit MDMA/MDA or 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA stimulated DA uptake into hDAT expressing cells (data not shown). We speculate that exposure of the hDAT to MDA/MDMA/metabolite causes physical alterations in the transporter that render it insensitive to nomifensine. Consistent with this view, the effects of MDMA on DA uptake in striatal synaptosomes were insensitive to the DAT ligand 2-β-carbomethoxy-3-β-(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)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA stimulate the transport of DA into hSERT-expressing SK-N-MC cells, whilst 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)-α-MeDA and 2,5-*bis*(GSyl)-α-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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Figure Legends

Figure 1. Metabolism of MDA and MDMA; formation of thioether metabolites. MDA and MDMA are demethylenated by cytochrome(s) P450 to α-MeDA (I), which is readily oxidized to an *ortho*-quinone (II). The *ortho*-quinone is quickly scavenged by GSH forming 5-(GSyl)-α-MeDA (III), which is subsequently oxidized to the *ortho*-quinone thioether (IV), which reacts with a second molecule of GSH to form 2,5-*bis*(GSyl)-α-MeDA (V). 2,5-*bis*(GSyl)-α-MeDA may also be oxidized to the corresponding *ortho*-quinone thioether (VI). The quinone species (II, IV, and VI) are oxidants and electrophiles capable of oxidizing (A), and alkylating (B) critical cellular macromolecular targets.

Figure 2. Western analysis of hSERT- and hDAT-expression in transfected SK-N-MC cells. Transfected cells were harvested and proteins were run on an SDS-PAGE gel and blotted with polyclonal antibodies against the hSERT or hDAT.

Figure 3. MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA inhibit 5-HT uptake into hSERT-transfected SK-N-MC cells. Cellular [^{3}H]5-HT uptake was determined in control (■), MDA (100 μM, △), MDMA (100 μM, ▼), 5-(GSyl)-α-MeDA (100 μM, ○) or 2,5-*bis*(GSyl)-α-MeDA (100 μM, ●) treated SK-N-MC cells at various times (0.5, 1, 2, 4, 8, 16, 24, and 48 hrs) by liquid scintillation spectroscopy. Data are plotted as the % specific (fluoxetine inhibitable) 5-HT uptake and expressed as the mean (n = 4) ± SEM. Differences between; i) control and treatment groups (*) and, ii) MDMA/MDA and metabolite groups (†) are considered significant at $p < 0.05$.

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Figure 4. Effects of fluoxetine and nomifensine on MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA-mediated inhibition of 5-HT uptake. Cellular [^{3}H]5-HT uptake was measured 4 hrs following treatment of hSERT-transfected cells. Control (black bars), MDA (100 μM; open bars), MDMA (100 μM; grey bars), 5-(GSyl)-α-MeDA (100 μM; gradient bars) or 2,5-*bis*(GSyl)-α-MeDA (100 μM; hatched bars). Groups of cells were incubated with fluoxetine (100 μM) or nomifensine (50 μM) 20 min prior to treatment. Data are presented as the % inhibition of 5-HT uptake and are expressed as the mean (n=4) ± SEM and differences between; i) control and treatment groups (*) and, ii) MDMA/MDA and metabolite groups (†) are considered significant at $p<0.05$. (§) Significantly different from untreated and nomifensine treated groups at $p< 0.05$.

Figure 5. Kinetics of MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA-mediated inhibition of 5-HT uptake. A. Specific [^{3}H]5-HT uptake was determined by saturation analysis, by treating SK-N-MC cells with increasing concentrations of [^{3}H]5-HT either alone or in the presence of fluoxetine (10 μM) for 30 min, measuring the intracellular accumulation of [^{3}H]5-HT, and taking the difference in uptake between non-specific and total 5-HT uptake. K_m and V_{max} represent mean values of 4 independent transfections. **B.** Ki values were determined by incubating cells with 20 nM [^{3}H]5-HT and increasing concentrations of MDA (□100 μM, ■), MDMA (□100 μM, □), 5-(GSyl)-α-MeDA (□100 μM, ●), or 2,5-*bis*(GSyl)-α-MeDA (□100 μM, ○) prior to

measuring intracellular [^{3}H]5-HT accumulation. The data were analyzed by non-linear least squares curve fit using a Km value of .3192 and plotted as the % specific 5-HT uptake. Ki values are; MDA = 107 μM, MDMA = 102 μM, 5-(GSyl)-α-MeDA = 69 μM, and 2,5-*bis*(GSyl)-α-MeDA = 49 μM.

Figure 6. MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA-induce ROS generation in hSERT-transfected cells. ROS generation was determined in hSERT (A) and hDAT (B) transfected SK-N-MC cells by loading the cells with DCF-DA and incubating control (■), MDA (100 μM, △), MDMA (100 μM, ▼), 5-(GSyl)-α-MeDA (100 μM, ○) or 2,5-*bis*(GSyl)-α-MeDA (100 μM, ●) samples for various periods of time (0.5, 1, 4, 8, 16, and, 24 hrs). Data are expressed as the mean (n=4) ± SEM. Differences between; i) control and treatment groups (*) and, ii) MDMA/MDA and metabolite groups (†) are considered significant at $p < 0.05$.

Figure 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, 200, 400 μM) of the compounds and by measuring increases in DCF fluorescence 4 hrs following treatment. Control (■), MDA (△), MDMA (▼), 5-(GSyl)-α-MeDA (○) or 2,5-*bis*(GSyl)-α-MeDA (●). Data are expressed as the mean (n=4) ± SEM and differences between; i) control and treatment groups (*) and, ii) MDMA/MDA and metabolite groups (†) are considered significant at $p < 0.05$.

Figure 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 hr incubation with the compounds by measuring changes in DCF-DA fluorescence. Vehicle control (black bars), MDA (100 μM; open bars), MDMA (100 μM; grey bars), 5-(GSyl)-α-MeDA (100 μM; hatched bars) or 2,5-*bis*(GSyl)-α-MeDA (100 μM; gradient bars) data are presented as the % increase in DA uptake. Groups of cells were incubated with fluoxetine (100μM) or nomifensine (50μM) 20 min prior to treatment. Data are expressed as the mean (n=4) ± SEM and differences between; i) vehicle control and treatment groups (*) and, ii) MDA/MDMA and metabolite groups (†) are considered significant at $p < 0.05$. (§) Significantly different from untreated and nomifensine treated groups at $p< 0.05$.

Figure 9. MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-*bis*(GSyl)-α-MeDA-induce DA uptake into hSERT-expressing SK-N-MC cells. Cellular [^{3}H]-DA uptake was determined in control (■), MDA (100 μM, △), MDMA (100 μM, ▼), 5-(GSyl)-α-MeDA (100 μM, ○) or 2,5-*bis*(GSyl)-α-MeDA (100 μM, ●) treated SK-N-MC cells at various times (0.5, 1, 2, 4, 8, 16, 24, and 48 hrs) by liquid scintillation spectroscopy. Data are expressed as the mean (n=4) ± SEM. Differences between; i) control and treatment groups (*) and, ii) metabolite groups and MDA/MDMA (†) are considered significant at $p < .05$.

Figure 10. Fluoxetine, but not nomifensine attenuates MDA, MDMA, 5-(GSyl)-α-MeDA and 2,5-bis(GSyl)-α-MeDA-stimulated DA uptake in hSERT-expressing SK-N-MC cells. Cellular [^{3}H]-DA uptake was determined in hSERT-transfected SK-N-MC cells by liquid scintillation spectroscopy 4 hrs following treatment. Control (black bars), MDA (100 μM; open bars), MDMA (100 μM; grey bars), 5-(GSyl)-α-MeDA (100 μM; gradient bars) or 2,5-*bis*(GSyl)-α-MeDA (100 μM; hatched bars) data are presented as the % increase of [^{3}H]-DA uptake. Groups of cells were incubated with fluoxetine (100 μM) or nomifensine (50 μM) 20 min prior to treatment. Data are expressed as the mean (n=4) ± SEM and differences between; i) control and treatment groups (*) and, ii) MDA/MDMA and metabolite groups (†) are considered significant at $p < 0.05$. (§) Significantly different from untreated and nomifensine treated groups at $p< 0.05$.

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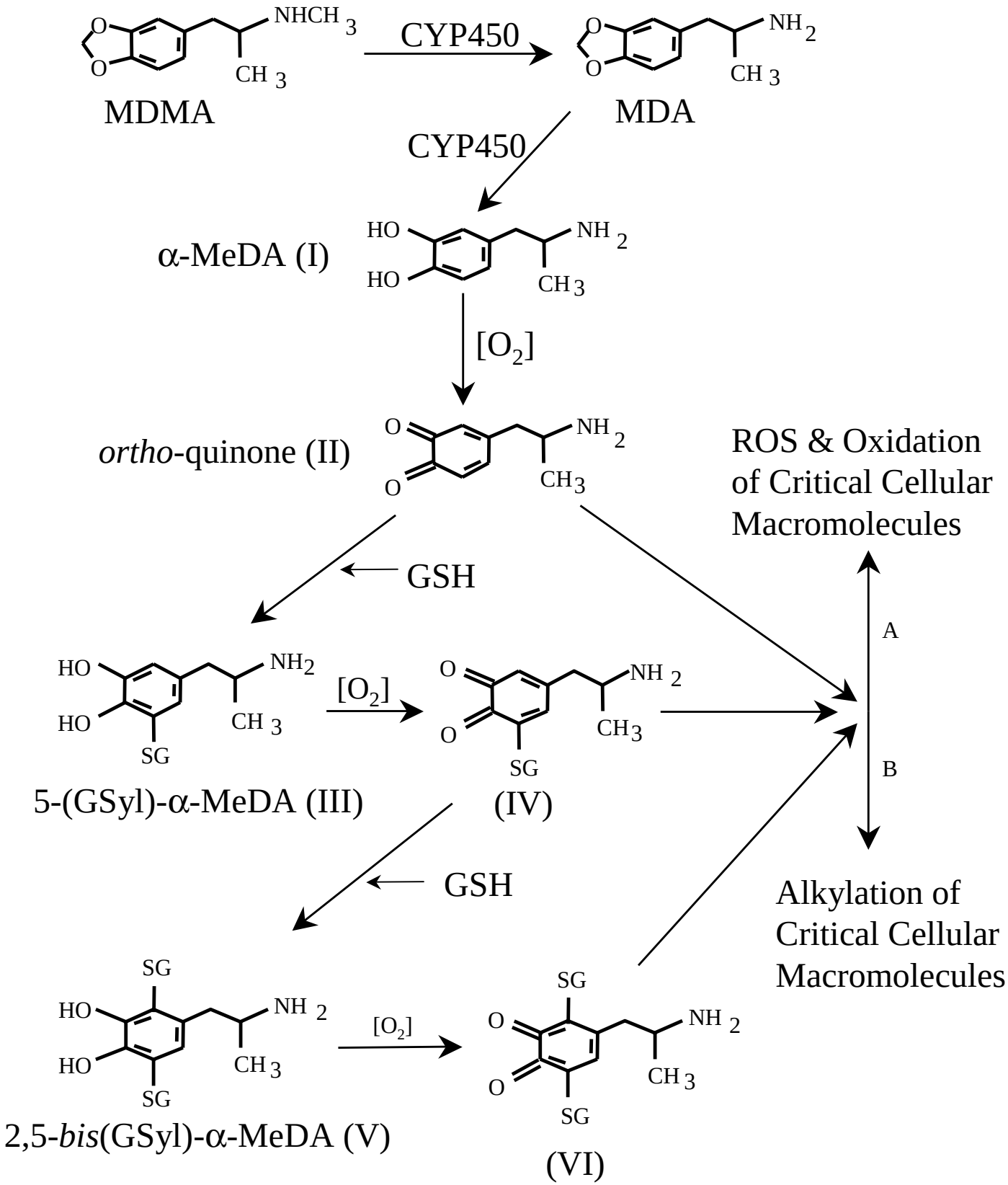

Figure 1

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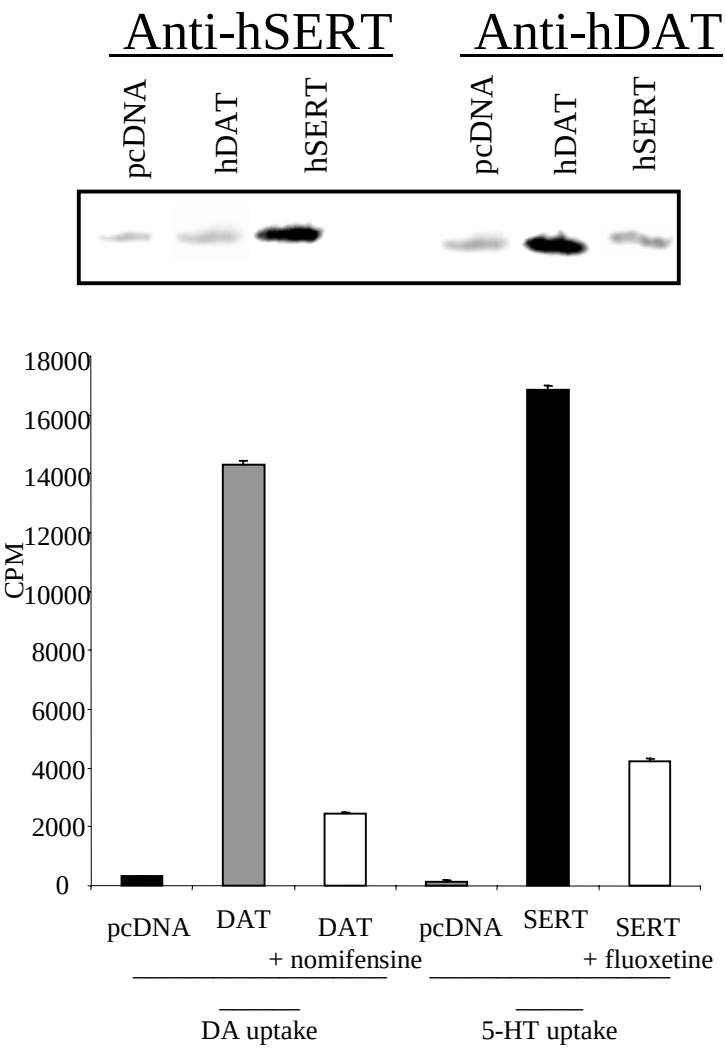

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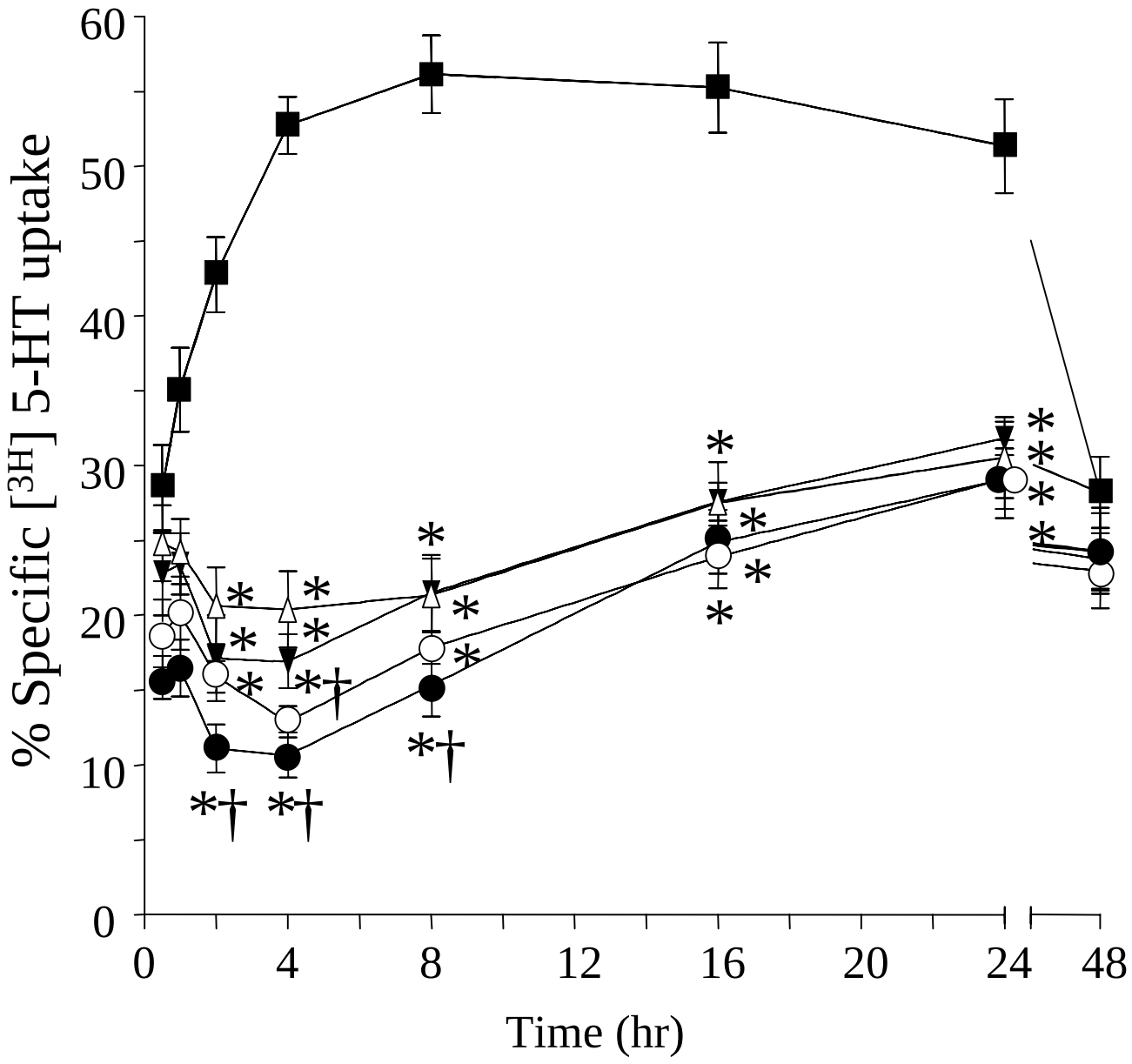

Figure 3

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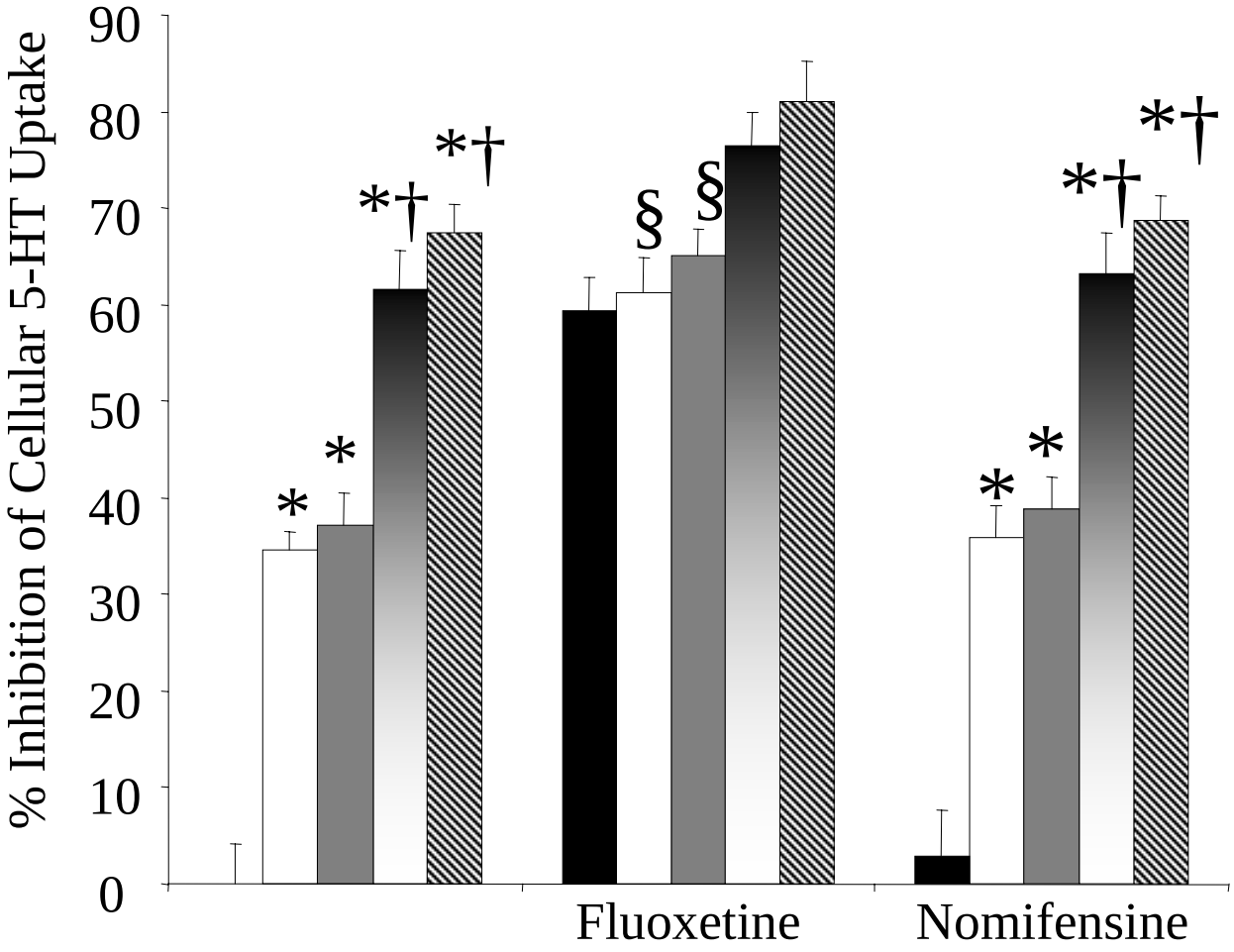

Figure 4

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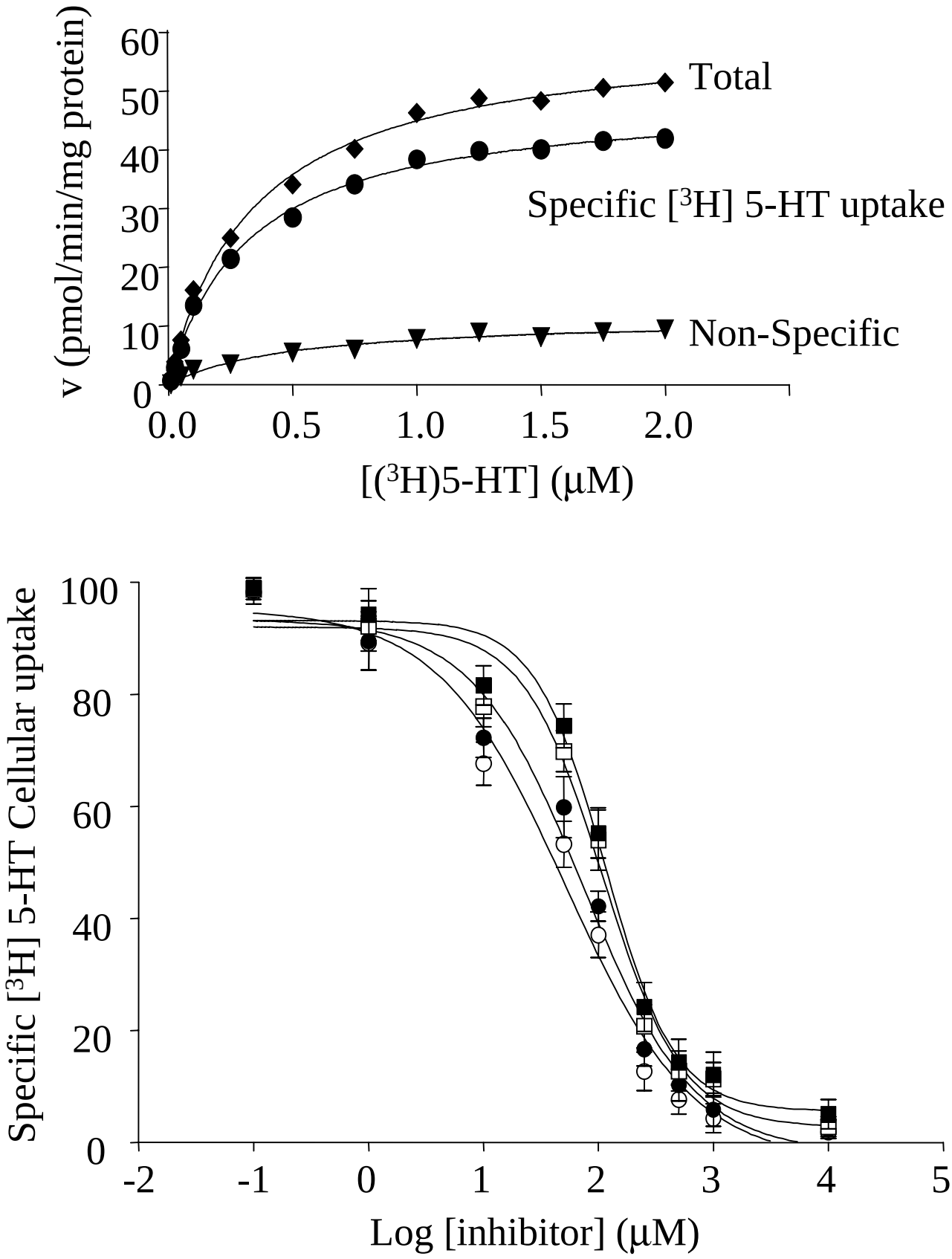

Figure 5

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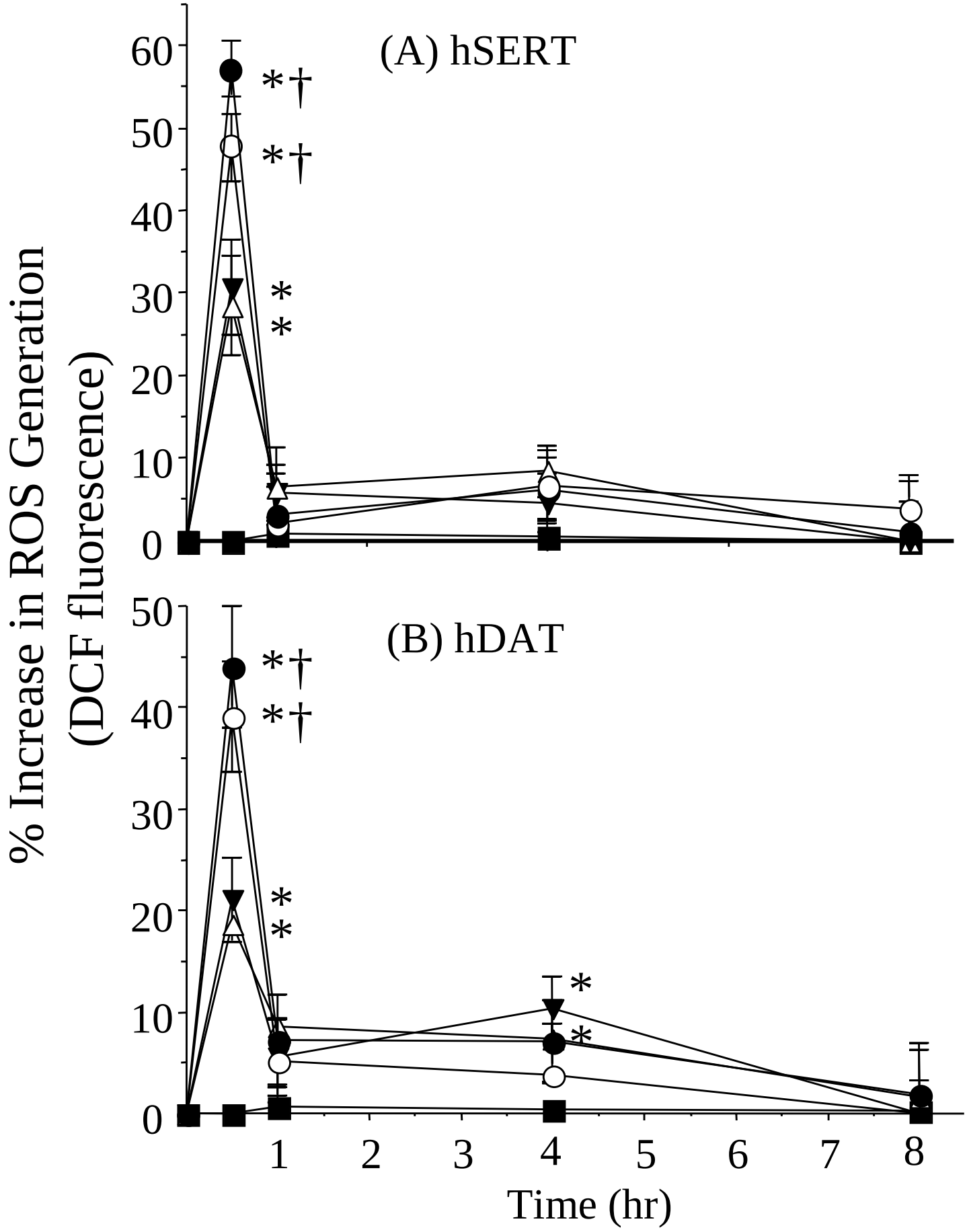

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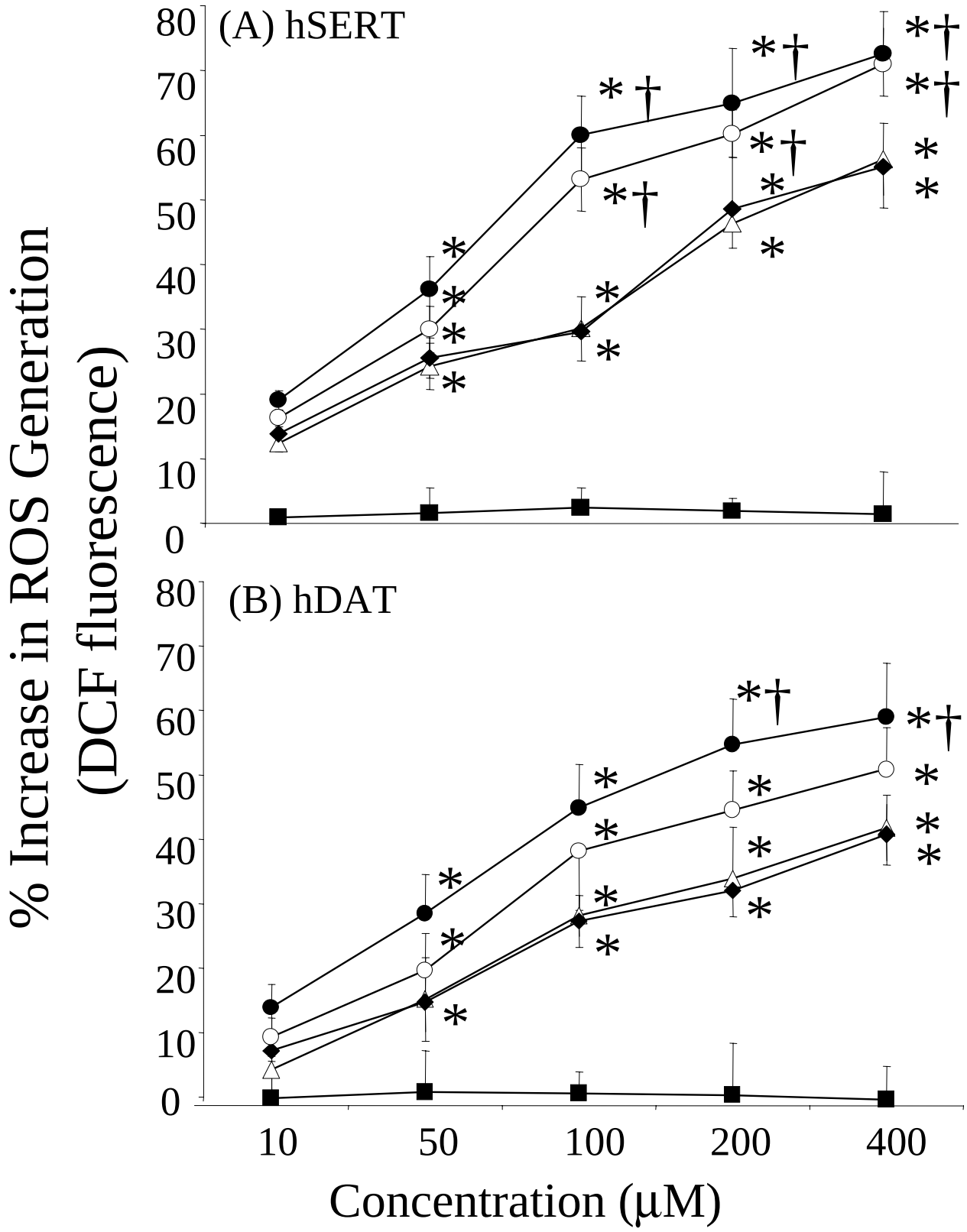

Figure 7

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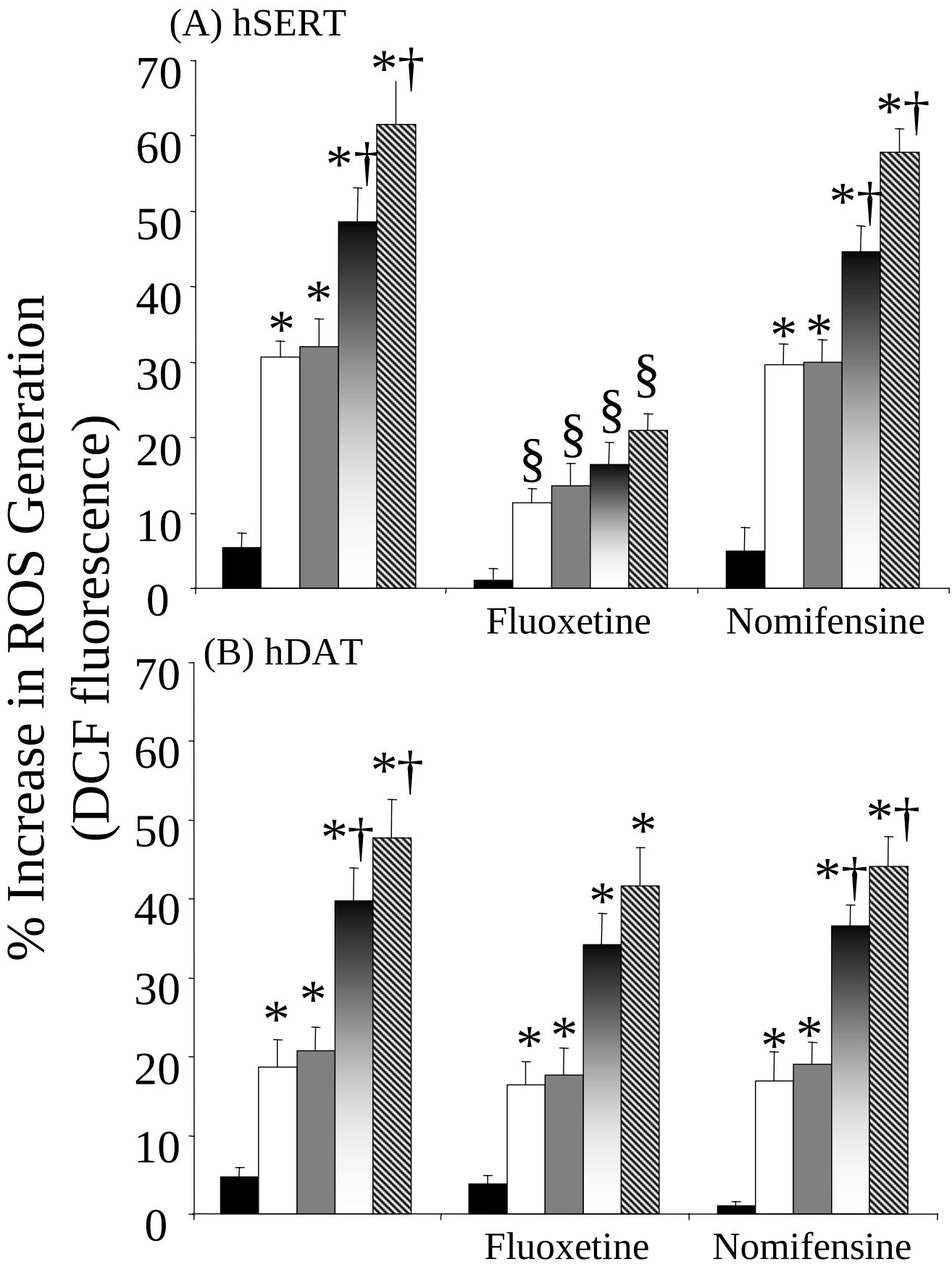

Figure 8

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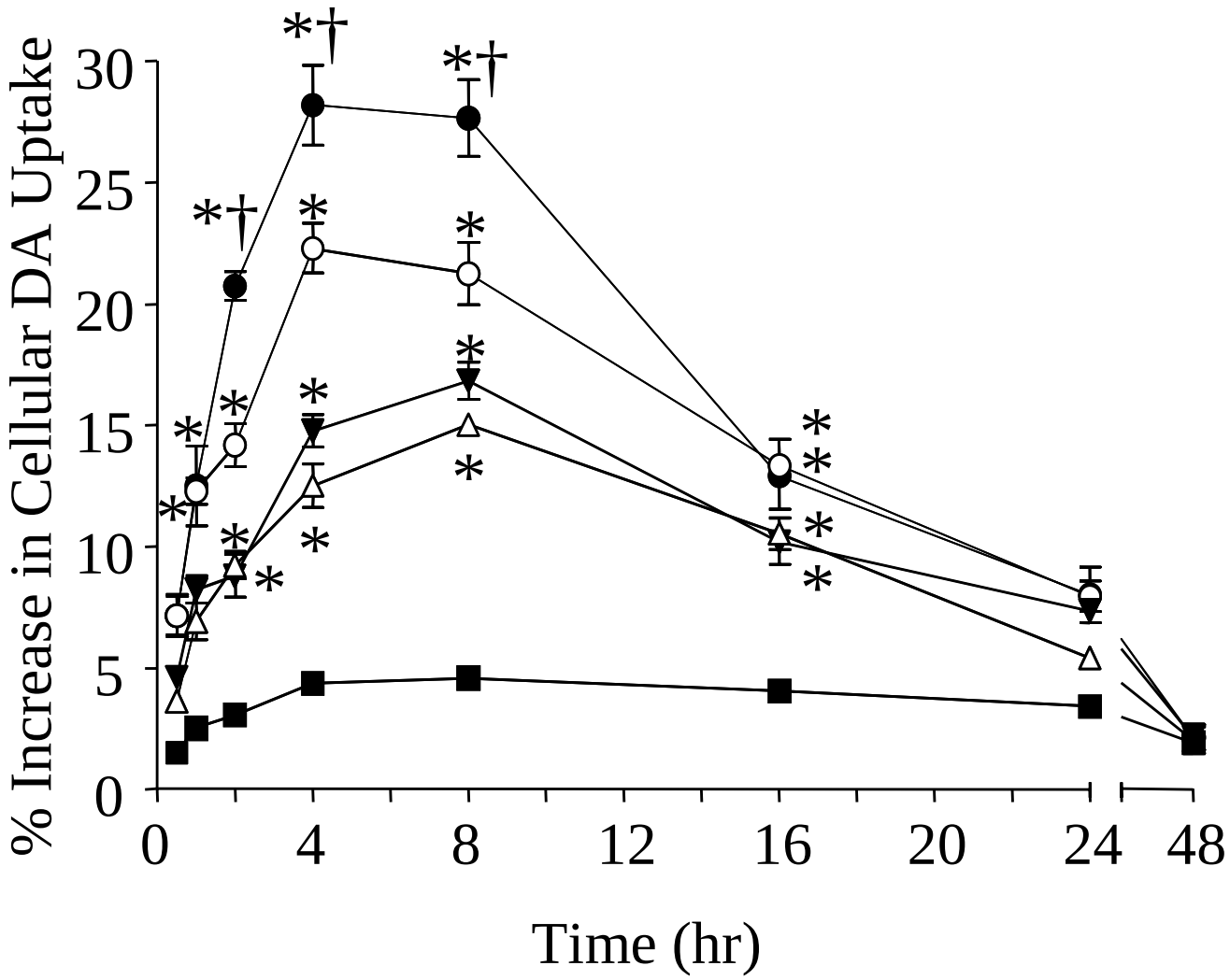

Figure 9

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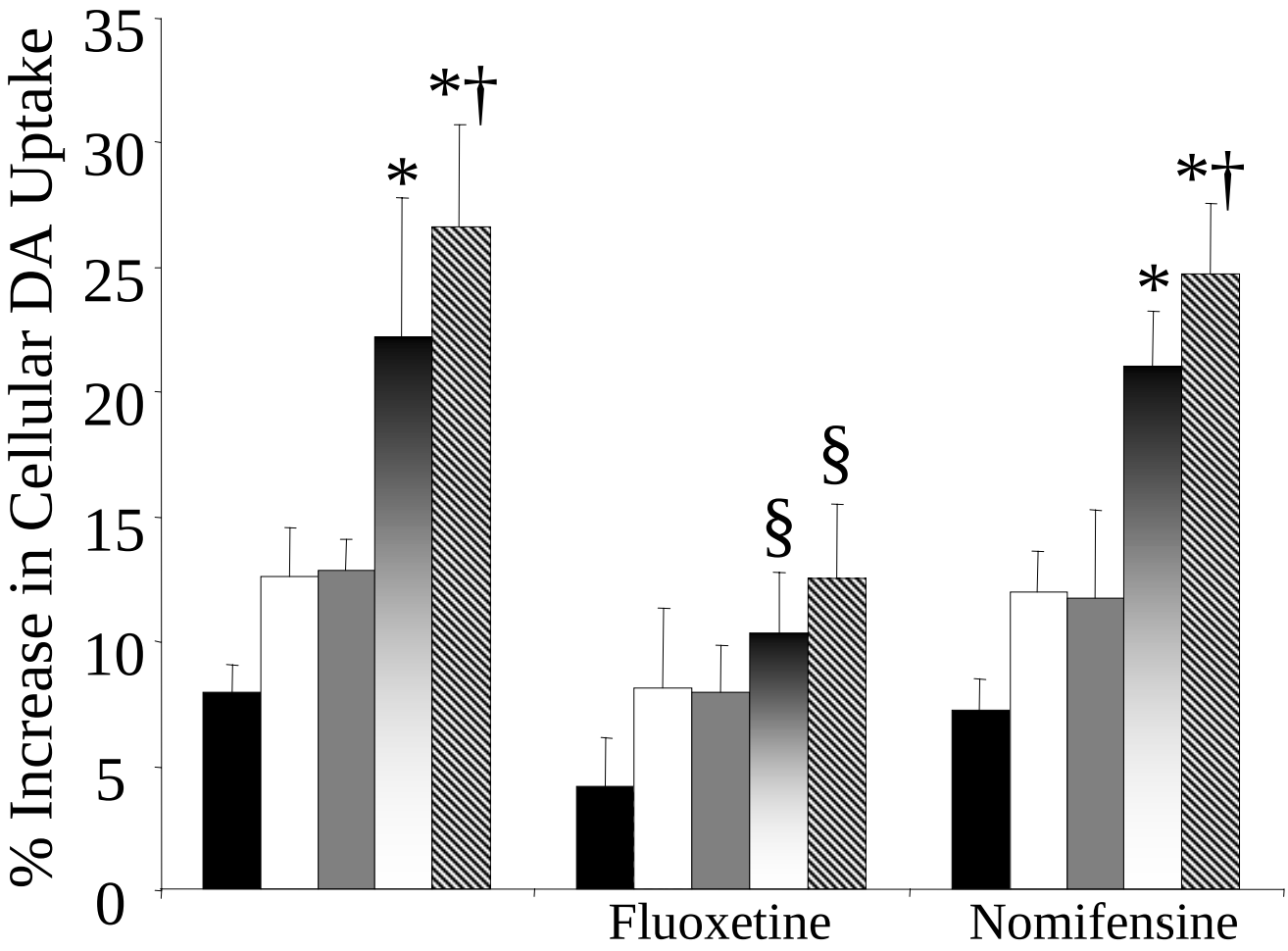

Figure 10