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Effect of repeated exposure to MDMA on the function of the 5-HT transporter as assessed by synaptosomal 5-HT uptake

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Abstract

Recent studies have demonstrated that a preconditioning regimen (i.e., repeated low doses) of MDMA provides protection against the reductions in tissue concentrations of 5-HT and 5-HT transporter (SERT) density and/or expression produced by a subsequent binge regimen of MDMA. In the present study, the effects of preconditioning and binge treatment regimens of MDMA on SERT function were assessed by synaptosomal 5-HT uptake. Synaptosomal 5-HT uptake was reduced by 72% 7 days following the binge regimen (10 mg/kg, ip every 2 hr for a total of 4 injections). In rats exposed to the preconditioning regimen of MDMA (daily treatment with 10 mg/kg for 4 days), the reduction in synaptosomal 5-HT uptake induced by a subsequent binge regimen was significantly less. Treatment with the preconditioning regimen alone resulted in a transient 46% reduction in 5-HT uptake that was evident 1 day, but not 7 days, following the last injection of MDMA. Furthermore, the preconditioning regimen of MDMA did not alter tissue concentrations of 5-HT, whereas the binge regimen of MDMA resulted in a long-term reduction of 40% of tissue 5-HT concentrations. The distribution of SERT immunoreactivity (ir) in membrane and endosomal fractions of the hippocampus also was evaluated following the preconditioning regimen of MDMA. There was no significant difference in the relative distribution of SERTir between these two compartments in control and preconditioned rats. The results demonstrate that SERT function is transiently reduced in response to a preconditioning regimen of MDMA, while long-term reductions in SERT function occur in response to a binge regimen of MDMA. Moreover, a preconditioning regimen of MDMA provides protection against the long-term reductions in SERT function evoked by a subsequent binge regimen of the drug. It is tempting to speculate that the neuroprotective effect of MDMA preconditioning results from a transient down-regulation in SERT function.

Keywords

MDMA; serotonin; serotonin transporter

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Conflict of Interest Statement:

The authors declare no conflicts of interest.

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA) or 'ecstasy' is a popular drug of abuse which exhibits both psychostimulant and mild hallucinogenic properties (Shulgin, 1986). MDMA is considered to be selectively neurotoxic to serotonergic neurons and is known to cause significant reductions in tryptophan hydroxylase activity (Schmidt and Taylor, 1987), serotonin (5-HT) tissue concentration (Stone et al., 1986), and 5-HT uptake sites (Battaglia et al., 1987).

While the mechanism of MDMA neurotoxicity is not fully understood, it is hypothesized that oxidative stress plays a key role in this process. Previous studies have shown that MDMA increases free radical formation (Colado and Green, 1995; Shankaran et al., 1999) and nitric oxide production (Darvesh et al., 2005) while decreasing endogenous antioxidants (Shankaran et al., 2001). Furthermore, the administration of antioxidants attenuates MDMA-induced neurotoxicity which lends support to this hypothesis (Colado and Green, 1995; Gudelsky, 1996; Shankaran et al., 2001). The 5-HT transporter (SERT) also appears to contribute to the process of neurotoxicity produced by MDMA. Indeed, studies have shown that fluoxetine attenuates MDMA-induced 5-HT release (Gudelsky and Nash, 1996) and free radical formation (Shankaran et al., 1999), as well as the long-term depletion of brain 5-HT (Aguirre et al., 1998; Malberg et al., 1996; Schmidt, 1987; Shankaran et al., 2001). Furthermore, it has been shown that SERT-KO mice are protected from MDMA-induced 5-HT depletions (Renoir et al., 2008).

Previous reports indicate that intermittent exposure to MDMA (i.e., preconditioning) prevents the long-term depletion of brain 5-HT elicited by a subsequent binge regimen of the drug (Bhide et al., 2009; Piper et al., 2006). However, the mechanisms underlying this neuroprotective phenomenon of chemical preconditioning remain to be elucidated. One possibility is that repeated exposure to MDMA, a SERT substrate, results in the down-regulation or internalization of SERT. Indeed, it has been shown that MDMA can induce a rapid decrease in SERT expression at the cell surface and a concurrent increase in intracellular expression in cell culture (Kivell et al., 2010). In view of the apparent necessity of SERT function for MDMA-induced 5-HT neurotoxicity, MDMA-induced down-regulation or internalization of SERT could contribute towards the neuroprotection afforded by preconditioning. Peraile et al. (2010) have proposed a similar mechanism involving the down-regulation of the dopamine transporter (DAT) to account for the finding that cocaine preconditioning confers protection against the MDMA-induced depletion of dopamine in the mouse.

In this study the effect of exposure to a preconditioning or binge regimen of MDMA on SERT expression and function was evaluated. SERT function was assessed by evaluating 5-HT uptake in synaptosomal preparations.

2. Materials and Methods

2.1 Animals

Adult male rats (250–350 g) of the Sprague–Dawley strain (Harlan Laboratories, Indianapolis, IN) were used in the studies and were housed two per cage at 23°C with an alternating 12 h light/dark cycle. Food and water were available ad libitum. Animals were sacrificed by decapitation. All procedures were in strict adherence to the National Institute of Health guidelines and approved by the Institutional Animal Care and Use Committee.

2.2 Drugs and Drug treatments

Racemic MDMA (National Institute on Drug Abuse, Rockville, MD) was dissolved in 0.15 M NaCl and administered i.p. The preconditioning regimen consisted of a daily injection of MDMA (10 mg/kg, i.p.) or vehicle for four days. Rats were sacrificed by decapitation either one or seven days after the last preconditioning injection. Rats receiving the binge regimen of MDMA were injected with MDMA, 10 mg/kg, i.p., at 2 h intervals for a total of 4 injections, and were sacrificed seven days after the last injection.

2.3 Synaptosomes preparation and novel microdialysis technique

Fresh hippocampal tissue was homogenized in 20 volumes of ice-cold 0.32M sucrose and centrifuged ($800 \times g$ for 24 minutes at 4°C). The supernatant was centrifuged at $22,000 \times g$ for 17 minutes at 4°C and the resulting pellet was resuspended in 20 volumes of ice-cold Dulbecco's phosphate buffered saline to produce the synaptosomal fraction.

A microdialysis technique was utilized that allows *in vitro* examination of extrasynaptosomal changes in the concentration of exogenously added 5-HT. An aliquot (285 μL) of the isolated synaptosomal fraction was incubated in the presence or absence of fluoxetine (10 μM) for 10 minutes at 30°C . Microdialysis probes were lowered into tubes upon the addition of 5-HT (35 μL) for a final concentration of 50nM. The loop-style probes were constructed from microbore PTFE tubing, PE-20 tubing, stainless steel wire, and Hospal AN69 HF membrane and had an active membrane length of 16mm. The probes were connected to an infusion pump which delivered modified Krebs Ringer's solution (2mM KCl, 1mM KH_2PO_4 , 1.2mM Ca_2Cl , 6.0 Na_2HPO_4 , 136mM NaCl) and 5mM glucose at a constant rate of 20 $\mu\text{L}/\text{min}$. Following the addition of 5HT and an incubation period of 18 minutes, three samples (40 μL each) were collected and the concentration of 5-HT in dialysis samples was determined using HPLC. A similar microdialysis technique has been employed to quantify vesicular glutamate uptake *in vitro* (Mark et al., 2007).

The mean 5-HT concentration of the samples was adjusted for probe recovery. The average probe recovery was 12.5%. The synaptosomal uptake of 5-HT was equated to the loss of 5-HT from the medium. Specific 5-HT uptake was calculated from the difference in uptake in the presence and absence of fluoxetine and was expressed as pg 5-HT/mg protein. Protein in the synaptosomal preparation was quantified by a Bradford assay.

2.4 Determination of tissue 5-HT concentrations using HPLC

Animals were killed by decapitation, and the hippocampus was isolated and stored at -80°C . Tissue samples were homogenized in 50 volumes of ice-cold 0.2 N perchloric acid and centrifuged at 14,000 rpm for 5 minutes. Aliquots (20 μL) of the supernatant were analyzed for 5-HT content by HPLC. Samples (20 μL) were injected onto a C-18 reverse phase column connected to an electrochemical detector (Coulchem II detector, ESA, Inc. Chelmsford, MA). The mobile phase consisted of 100 mM citric acid, 75 mM sodium phosphate, 50 mg/l disodium ethylenediamine tetraacetate, 176 mg/l octane sulfonic acid sodium salt, 16.5% methanol, pH 4.5, pumped at a flow rate of 0.9 ml/min. The potentials of E1 and E2 on the analytical cell were -160 and $+205$ mV, respectively. Peak heights were recorded on an integrator and the quantity of 5-HT was calculated based on known standards.

2.5 Determination of SERT expression using Western blot technique

Western blots were performed for the analysis of 5-HT (SERT) transporter expression in rats treated with either the preconditioning regimen of MDMA or vehicle and sacrificed by decapitation one day after the last injection. The hippocampus was isolated and stored at -80°C . Hippocampal tissue was homogenized in 20 mM sodium phosphate buffer; pH 7.4

containing 0.32M sucrose. The homogenate was centrifuged at $20,000 \times g$ for 20 min at 4°C . The supernatant was retained and the pellet (P1 membrane fraction) was washed twice in 0.3ml of homogenization buffer and centrifuged. The supernatants were combined, and centrifuged at $200,000 \times g$ for 60 min at 4°C to obtain the endosome pellet. The P1 and endosome pellet were finally resuspended in 15 volumes of the homogenization buffer.

Protein values of the hippocampal membrane pellets were determined via the Bradford method. Protein samples were mixed with Laemmli loading buffer, heated to 85°C and stored at -80°C until analysis by Western blot on 9% Tris-glycine gels. Protein (30 μg) was loaded into each lane and the gel was run at 50 milliamps for 3 hrs (running buffer: 25mM Tris; 192mM Glycine; .02% SDS). Gels were transferred at 100V for 1hr to a polyvinylidene difluoride (PVDF) membrane (transfer buffer: 25mM Tris; 192mM Glycine; 20% methanol; .02% SDS). The membranes were blocked for 1hr in blocking buffer (10mM Tris; 150mM NaCl; pH8.0; 5% non-fat dry milk and 0.2% Tween-20) and probed with anti-SERT primary antibody (dilution 1:250; Santa Cruz sc-33724, CA, USA) overnight at 4°C . The membranes were rinsed three times in TBST, probed with HRP-conjugated goat anti-mouse secondary antibody (dilution 1:5000) for 1hr, and visualized via enhanced chemiluminescence (ECL). The protein bands were quantified using the LAS-4000 mini luminescent analyzer (FujiFilm, Stamford, CT) and normalized to the actin signal on the Western blot.

2.6 Statistical analysis

Data were compared using a one-way or two-way ANOVA (SigmaStat, Jandel Scientific). Multiple pairwise comparisons were performed using the Student Newman-Keuls test. Treatment differences were considered statistically significant at $P < 0.05$.

3. Results

3.1 Effect of MDMA preconditioning on subsequent MDMA reduction in 5-HT uptake

A binge regimen of MDMA resulted in a greater than 70% reduction ($p < 0.001$) in synaptosomal uptake of 5-HT 7 days following treatment when compared to the values for control (vehicle) animals (Fig. 1). However, the binge regimen of MDMA did not significantly reduce synaptosomal 5-HT uptake in rats exposed to the preconditioning regimen of MDMA (10 mg/kg, ip daily for 4 days). Synaptosomal 5-HT uptake in rats given the binge regimen of MDMA was significantly ($p < 0.01$) greater in rats previously exposed to the preconditioning regimen of MDMA than in those preconditioned with vehicle. Although synaptosomal 5-HT uptake was slightly reduced 8 days following treatment with the preconditioning regimen alone, the reduction was not significant ($p = 0.09$). A two-way ANOVA revealed a significant effect of treatment [$F(1, 22) = 14.73$, $p < 0.001$] and a significant pretreatment $\times$ treatment interaction [$F(1, 22) = 11.37$, $p = 0.003$]. The factor of pretreatment was not significant [$F(1, 22) = 0.59$, $p = 0.45$].

3.2 Effect of the MDMA preconditioning regimen on 5-HT uptake

The preconditioning regimen of MDMA did produce a transient reduction in 5-HT uptake (Fig. 2). Synaptosomal 5-HT uptake was reduced by 45% 24 hrs following the last of the daily MDMA treatments. However, there was no difference in 5-HT uptake 7 days following the last daily injection of MDMA when compared to uptake in control animals. Two-way ANOVA revealed a significant effect of treatment [$F(1, 30) = 8.93$, $p < 0.01$]. Post-hoc analysis indicated that the MDMA preconditioning regimen reduced ($p < 0.01$) 5-HT uptake at 1 day, but not 7 days, following treatment.

3.3 Effect of binge and preconditioning regimens of MDMA on tissue 5-HT concentrations

Concentrations of 5-HT in the hippocampus were determined 7 days following the binge regimen of MDMA and 1 and 7 days following the last of the preconditioning treatments. Whereas the binge regimen of MDMA resulted in a 40% reduction in tissue concentrations of 5-HT at 7 days following treatment, concentrations of 5-HT were unaffected at either 1 or 7 days following the preconditioning regimen (Fig. 3). One-way ANOVA revealed a significant effect of treatment [$F(3,26)=17.74$, $p<0.001$], and post-hoc analysis revealed a significant ($p<0.001$) difference between the vehicle- and binge-treated animals, as well as ($p<0.001$) for the comparison between preconditioned (1 or 7 days) animals and binge-treated animals.

3.4 Effect of the preconditioning regimen of MDMA on the expression of SERT in sub-cellular fractions in the hippocampus

In order to determine whether the reduction in 5-HT uptake following the preconditioning regimen of MDMA is accompanied by an intracellular redistribution of SERT, SERT expression was quantified in membrane and endosomal fractions of hippocampal homogenates (Fig. 4). Analysis by ANOVA of the quantification of SERT immunoreactivity indicated no significant difference between control and MDMA preconditioned animals in either the membrane ($p=0.42$) or endosome ($p=0.5$) fractions.

4. Discussion

The key findings of the present study are that 1) a binge regimen of MDMA produces a long-term reduction in synaptosomal 5-HT uptake, 2) a preconditioning regimen of MDMA provides protection against the reduction in 5-HT uptake evoked by a subsequent binge regimen of MDMA, 3) a preconditioning regimen of MDMA alone produces a transient reduction in synaptosomal 5-HT uptake and 4) the distribution of SERTir between membrane and endosomal compartments is not significantly altered by the preconditioning regimen of MDMA.

The repeated administration of high doses of MDMA (i.e., binge regimen) has consistently been shown to produce persistent reductions in biochemical markers of 5-HT axon terminals (Schmidt, 1987; Xie et al., 2006; Yamamoto et al., 2010). In this regard, MDMA produces long-term reductions in brain concentrations of 5-HT and 5-HIAA, 5-HT fiber density, SERT binding density, SERTir, and tryptophan hydroxylase activity (Green et al., 2003; Yamamoto et al., 2010), and the reduction in these biochemical markers has generally been viewed as evidence of MDMA-induced 5-HT neurotoxicity. In the present study, a binge regimen of MDMA resulted in a long-term reduction in synaptosomal 5-HT uptake. Hence, not only is SERT binding density and SERTir reduced by a binge regimen of MDMA administration, but SERT function, as evidenced by 5-HT uptake in the present study, also is reduced. The persistent reduction in synaptosomal 5-HT uptake produced by binge MDMA administration may be the consequence of either a reduction in the density of 5-HT axon terminals (i.e., neurotoxicity) or a long-lasting down-regulation of SERT gene expression (Biezonski and Meyer, 2010; Biezonski and Meyer, 2011).

In previous studies, prior exposure of rats to intermittent MDMA (i.e., preconditioning) has been shown to provide neuroprotection against the subsequent reduction in the hippocampal concentration of 5-HT and in the density of SERT binding sites and SERTir produced by a binge regimen of MDMA (Bhide et al., 2009; Piper et al., 2006; Piper et al., 2010). The results of the present study are in accord with these earlier reports in that a preconditioning regimen of MDMA afforded protection against the reduction in synaptosomal 5-HT uptake evoked by a subsequent binge regimen of MDMA. The present data, taken together with the

aforementioned earlier reports, provide additional support for the view that prior intermittent exposure to MDMA provides protection against MDMA-induced 5-HT neurotoxicity.

The neuroprotective effects of MDMA preconditioning may involve mechanisms similar to those involved in ischemic preconditioning (Kitagawa et al., 1991), such as activation of ion channels, increased activity of antioxidant or anti-apoptotic enzymes, or increased heat shock proteins. However, another potential mechanism whereby MDMA preconditioning provides neuroprotection may be through the down-regulation of SERT function. SERT function is critical for the process of MDMA-induced 5-HT neurotoxicity, as evidenced by the fact that fluoxetine, an inhibitor of SERT function, attenuates MDMA-induced 5-HT depletion (Malberg et al., 1996; Schmidt, 1987; Shankaran et al., 1999). In the present study, the preconditioning regimen of MDMA resulted in a transient down-regulation of SERT function, as evidenced by a 45% reduction in synaptosomal 5-HT uptake. This reduction occurred 1 day following the preconditioning regimen, a time at which the binge regimen of MDMA was administered. It seems likely that the reduction in 5-HT uptake at this 1 day time point reflects a transient down-regulation of SERT function rather than a loss of terminals through neurotoxicity since neither 5-HT uptake nor 5-HT tissue concentrations were reduced at 7 days following preconditioning, as was the case following the binge regimen of MDMA. The down-regulation of SERT function by MDMA preconditioning may provide neuroprotection by limiting the involvement of SERT in the dopamine-, MDMA- or 5-HT-dependent generation of reactive oxygen species.

SERT-dependent actions of MDMA have been proposed by some investigators to involve the actions of metabolites of MDMA, e.g., thioether conjugates of α -methyldopamine and N-methyl- α -methyldopamine (Jones et al., 2004). In the present study, we cannot exclude the possibility that MDMA metabolites mediate either the persistent reduction in SERT function produced by the binge regimen of MDMA or the transient reduction in SERT function elicited by the preconditioning regimen.

In the present study, the down-regulation of SERT function was evident 1 day, but not 7 days, following the preconditioning regimen of MDMA, and the neuroprotective effect afforded by this preconditioning regimen has been shown to be evident at 1 day, but not 7 days (Bhide et al., 2009). However, Meyer and colleagues (Piper et al., 2006; Piper et al., 2010) have employed a more prolonged preconditioning regimen and demonstrated an accompanying period of neuroprotection of up to 1 week. It is presently unknown whether SERT function can be down-regulated for longer durations by different preconditioning regimens of MDMA.

Although the present study does not establish a causal relationship between the down-regulation of SERT and the preconditioning phenomenon, it is noteworthy that intermittent cocaine administration produces down-regulation of the dopamine transporter (DAT) in plasma membranes and protection against MDMA-induced dopamine depletion in the mouse (Peraile et al., 2010). In this study, Peraile et al. (2010) concluded that cocaine preconditioning provides protection against MDMA-induced dopamine neurotoxicity through a PKC-dependent internalization (i.e., downregulation) of DAT.

In vitro studies carried out by Kittler et al. (2010) indicate that exposure to 5-HT uptake inhibitors and other SERT substrates, such as 5-HT and MDMA, result in the internalization of SERT. Phosphorylation of SERT induces translocation to intracellular compartments, and SERT can be redistributed to the cell-surface upon activation by phosphatases (Steiner et al., 2008). Ramamoorthy and colleagues have demonstrated that PKC activators produce phosphorylation of SERT that parallels SERT internalization (Qian et al., 1997).

Interestingly, MDMA has been demonstrated to induce translocation and activation of PKC in the cortex and hippocampus (Kramer et al., 1997).

In the present study, the preconditioning regimen of MDMA did not alter the subcellular distribution of SERT, suggesting that SERT was not internalized by the preconditioning regimen of MDMA. One explanation for this discrepancy may be a difference in the regulation of SERT upon exposure to SERT substrates in vitro when compared to in vivo conditions. For example, German et al. (2012) noted that in vivo exposure of rats to amphetamine or methamphetamine did not result in internalization of DAT, despite findings that internalization occurs following exposure in vitro. Alternatively, MDMA preconditioning could result in free radical-mediated inactivation of SERT in the absence of SERT internalization. MDMA has been shown to promote the formation of reactive oxygen and nitrogen species (Colado et al., 1997; Shankaran et al., 1999; Yamamoto et al., 2010), and SERT has been shown to undergo oxidative inactivation without an alteration in ligand binding (Asano et al., 1997). However, we cannot exclude the possibility that there was a normalization of the distribution of SERT during the process of synaptosome preparation such that MDMA-induced internalization of SERT was no longer evident. Nevertheless, the findings of German and those in the present study suggest mechanisms in addition to internalization may contribute to the reduction in function of DAT or SERT following in vivo exposure to psychostimulant substrates.

Acknowledgments

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Highlights

1. A binge regimen of MDMA produces a long-term reduction in synaptosomal 5-HT uptake
2. MDMA preconditioning prevents the reductions in 5-HT uptake following binge MDMA
3. MDMA preconditioning produces a transient reduction in synaptosomal 5-HT uptake
4. MDMA preconditioning does not alter the subcellular distribution of SERTir.

Fig. 1. Effect of the preconditioning regimen on the subsequent MDMA-induced reduction in synaptosomal 5-HT uptake

Rats were treated with either the preconditioning regimen of MDMA (10 mg/kg i.p.) or vehicle once daily for four days. On the following day, the rats were injected with either the binge regimen of MDMA (10 mg/kg, i.p. at 2 h intervals for a total of 4 injections) or vehicle and were sacrificed seven days after the last injection by decapitation. The values represent the mean $\pm$ SE of 5–8 rats. * represents $p < 0.001$ when treatment groups are compared within the factor of prior exposure and # represents $p < 0.01$ when compared to MDMA binge-treated rats previously exposed to vehicle.

Fig. 2. Effect of the preconditioning regimen on synaptosomal uptake of 5-HT at 1 or 7 days after the last injection of MDMA

Rats were treated with the preconditioning regimen of MDMA (10 mg/kg i.p.) or vehicle once daily for four days. Rats were sacrificed by decapitation either one or seven days after the last injection. The values represent the mean $\pm$ SE of 7–11 rats. * represents $p < 0.01$ when compared to the corresponding vehicle-treated group.

Fig. 3. Comparison of the preconditioning and binge regimen of MDMA on tissue 5-HT concentrations in the hippocampus

Rats treated with the preconditioning regimen of MDMA (10 mg/kg i.p., once daily for four days) were sacrificed by decapitation either one (PC1) or seven days (PC7) after the last injection. Rats treated with binge regimen of MDMA (10 mg/kg, i.p. at 2 h intervals for a total of 4 injections) or vehicle were sacrificed seven days after the last injection. The values represent the mean $\pm$ SE of 5–10 rats. * represents $p < 0.001$ vs. the vehicle-treated group, as well as vs. the PC1 and PC7 groups.

Fig. 4. Effect of the preconditioning regimen of MDMA on hippocampal plasma membrane (a and c) and endosome (b and d) SERT expression
Rats were treated with the preconditioning regimen of MDMA (10 mg/kg i.p.) or vehicle once daily for four days and were sacrificed one day after the last injection by decapitation. The values represent the mean $\pm$ SE of 5–6 rats.