3,4-Methylenedioxymethamphetamine (MDMA) Administration to Rats Decreases Brain Tissue Serotonin but not Serotonin Transporter Protein and Glial Fibrillary Acidic Protein

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ABSTRACT Previous experiments conducted in this laboratory showed that administration of high-dose D-fenfluramine (D-FEN) and p-chloroamphetamine (PCA) decreased 5-HT transporter (SERT) binding and tissue 5-HT by 30–60% in caudate and whole brain tissue 2 days and 2 weeks after drug administration. However, protein expression as determined by Western blot analysis did not change in either tissue or time point, except for a 30% decrease in the caudate 2 days after PCA administration. In the present study, we studied the effect of MDMA and 5,7-dihydroxytryptamine (5,7-DHT) on tissue 5-HT levels and the protein expression level of SERT and glial fibrillary acidic protein (GFAP), a validated neurotoxicity marker. Hypothesis. MDMA administration decreases SERT expression. Methods. Two weeks after MDMA administration (7.5 mg/kg i.p., q 2 h × 3 doses) or 2 weeks after i.c.v. administration of 5,7-DHT (150 μg/rat), male Sprague-Dawley rats were sacrificed and the caudate, cortex, and hippocampal tissue collected. Western blots for SERT and GFAP were generated using published methods. Tissue 5-HT levels were determined by HPLC coupled to electrochemical detection. Results. MDMA treatment decreased tissue 5-HT in cortex, hippocampus, and caudate by about 50%. However, MDMA treatment had no significant effect on expression level of SERT and GFAP in any brain region. In contrast, 5,7-DHT reduced tissue 5-HT by more than 90%, decreased SERT protein expression by 20–35%, and increased GFAP by 30–39%. Conclusion. These data suggest the MDMA treatment regimen used here does not cause degeneration of 5-HT nerve terminals. Viewed collectively with our previous results and other published data, these data indicate that MDMA-induced persistent 5-HT depletion may occur in the absence of axotomy. Synapse 53:240–248, 2004. Published 2004 Wiley-Liss, Inc.

INTRODUCTION

The club drug 3,4-methylenedioxymethamphetamine (MDMA, “ecstasy”) is widely used as an illicit recreational drug. Numerous studies in rodents and nonhuman primates indicate that MDMA administration can produce long-lasting decreases in markers of the serotonergic nerve terminal (for review, see Lyles and Cadet, 2003; Sprague et al., 1998). These markers include reductions in tissue 5-HT levels, 5-HT transporter (SERT) binding and function (Commins et al., 1987; Schmidt, 1987), and tryptophan hydroxylase activity. Immunohistochemical analysis of 5-HT shows an apparent loss of 5-HT nerve terminals. The spectrum of decrements in serotonergic markers produced by MDMA administration is typically described as neurotoxicity. Despite there being over 60 articles report-
MDMA DOES NOT DECREASE SERT PROTEIN

In light of our unexpected results with D-fenfluramine and PCA, in the present study we administered MDMA according to a neurotoxic regimen and measured SERT and GFAP protein expression. As a control, we administered the neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) via the intracerebroventricular (i.c.v.) route in a separate group of animals. We hypothesized that both MDMA and 5,7-DHT would decrease SERT expression and increase GFAP expression. Unexpectedly, we observed that MDMA had no effect on either SERT or GFAP expression, despite large decreases in tissue 5-HT.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 280–320 g were singly housed (lights on: 0700–1900 h) with food and water freely available. Rats were maintained in facilities accredited by the American Association of the Accreditation of Laboratory Animal Care, and the procedures described herein were carried out in accordance with the Animal Care and Use Committee of the National Institute on Drug Abuse (NIDA) Intramural Research Program (IRP).

For MDMA-HCl treatments, rats received i.p. injections (7.5 mg/kg) every 2 h for three doses. For 5,7-DHT treatments, rats received a single i.c.v. injection of 150 µg in 10 µl vehicle (0.1% ascorbic acid in saline). Control rats received i.p. saline injections (1 ml/kg) or i.c.v. injections of 10 µl vehicle. All rats were sacrificed 2 weeks later. After sacrifice, the frontal cortex, caudate, and hippocampus were dissected. Tissue was kept frozen at −70°C until used in the Western blots.

We developed the MDMA dose and injection schedule according to the principles of “effect scaling,” rather than “interspecies scaling” (Ricaurte et al., 2000). Effect scaling involves the determination of drug doses required to evoke equivalent pharmacological responses in rats vs. humans. Doses of MDMA (~1.5 mg/kg) that are discriminated readily by rats (Baker and Makhay, 1996; Glennon and Higgs, 1992) are identical to those misused recreationally by humans (Cole and Sumnall, 2003). We therefore administered a dose of MDMA that is 5 times higher than the described dose 3 times in 6 h, an effective dose 15 times higher than the dose typically taken by a human. We chose a 2-week sacrifice time since this time point is commonly used in the literature as an appropriate time to measure long-term effects of MDMA.

Western blot analysis

Tissues were homogenized by sonication in cold RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxy-
cholate, 0.1% SDS, 1 mM PMSF, 10 mg/ml aprotinin, 1 mM sodium orthovanadate in PBS buffer, pH 7.4).

Protein concentrations were determined using Pierce BCA Protein Assay Reagent Kit (Rockford, IL) and homogenates were diluted to a concentration of 2 mg/ml with 2X SDS-PAGE loading buffer (Invitrogen, Carlsbad, CA). Samples were boiled for 6 min. Sample protein (6 μg/lane for GFAP and 60 μg/lane for SERT assay) was separated on 8–16% polyacrylamide mini-gels (Invitrogen). Proteins separated by electrophoresis were transferred to Immobilon-PVDF membranes (Millipore, Bedford, MA) using a semi-dry apparatus (Bio-Rad, Hercules, CA). Nonspecific binding to membranes was prevented by blocking for 60 min at room temperature in TBS solution containing 5% nonfat dry milk. Membranes were then probed by overnight incubation with a 1:500 dilution of GFAP antibody (RDI, Flanders, NJ) and a 1:100 dilution of SERT antibody (Calbiochem, La Jolla, CA). Membranes were rinsed three times with TBS, then incubated with a 1:5,000 dilution of horseradish peroxidase-labeled secondary antibody in TBS solution containing 0.25% nonfat dry milk for 90 min at room temperature. After washing three more times, antibody complex were visualized by chemiluminescence using a kit from Pierce Biotechnology.

Measurement of 5-HT

Rat cortical tissue levels of DA, DOPAC, NE, HVA, 5-HT, and 5-HIAA were measured using high-pressure liquid chromatography (HPLC) coupled to electrochemical detection as previously described (Baumann et al., 1998).

Experimental design, data analysis, statistics, and reagents

Two groups of rats were treated with MDMA (n = 7) or saline (n = 7) and a separate group of rats was treated with 5,7-DHT (n = 7) or vehicle (n = 7). Tissue 5-HT was measured in all animals in all three regions of the brain as the primary endpoint marker for the 5-HT nerve terminal. We did not assess the effect of MDMA treatments on SERT binding, because this endpoint decreases in tandem with tissue 5-HT (Green et al., 2003). With MDMA, we observed a broad range of 5-HT depletion. For example, following MDMA administration the range of 5-HT cortical depletion was 22–93%. To control for this variability, we measured SERT protein expression in two groups of MDMA-treated rats: three rats with an ~40% depletion of cortical 5-HT and two rats with an ~90% depletion of cortical 5-HT. The main purpose of the 5,7-DHT experiments was not to develop a positive control for the GFAP experiments, since it is already known that 5,7-DHT increases GFAP expression (Fages et al., 1994; Frankfurt et al., 1991; O’Callaghan and Miller, 1994). We wished to have a positive control for the SERT measurement, since our pilot studies with MDMA indicated that this drug did not decrease SERT expression.

Western blots were quantitated using standard methods (Jayanthi et al., 2002). Control and treated samples were run side-by-side. The density of the SERT band in the treated sample was divided by the density of the SERT band in the control sample and multiplied by 100 to yield a percent of control. This was done for each sample. The mean and SD of the percent of control was calculated using the results of these replicant samples. Statistical significance of the 5-HT tissue data and the Western blot data was determined by Student’s t-test. The sources of reagents used in this study are described in previous publications as cited above.

RESULTS

An important set of control experiments was to determine if the Western blot analyses using antibodies for SERT and GFAP could detect changes in the expression of these proteins. As reported in Figure 1, SERT and GFAP immunoreactivity linearly increased as a function of the amount of protein sample loaded onto the gel.
As noted above, two groups of rats were treated with MDMA (n = 7) or saline (n = 7) and a separate group of rats were treated 5,7-DHT (n = 7) or vehicle (n = 7). 5-HT was measured in three brain regions 2 weeks after drug administration. MDMA decreased 5-HT by 49.5 ± 9.9% (caudate), 50.2 ± 10.2% (hippocampus), and 55.5 ± 10.2% (cortex) (mean ± SD, n = 7) and 5,7-DHT decreased 5-HT by 70.7 ± 10.5% (caudate), 81.7 ± 7.4% (hippocampus), and 73.9 ± 8.9% (cortex) (n = 7). For the MDMA group, three rats with percent decreases of ~40% were selected for further study, and for the 5,7-DHT group four rats with percent decreases of about 90% were selected for further study (Fig. 2). MDMA treatment did not alter either SERT or GFAP expression (Fig. 3). In contrast, 5,7-DHT significantly decreased SERT expression and increased GFAP expression (Fig. 4). As an additional control, two MDMA-treated rats that had decrements of 5-HT in the range of 90% were also assayed for SERT and GFAP expression. Neither rat demonstrated GFAP or SERT expression different from control in the brain regions examined (Fig. 5).

**DISCUSSION**

Many studies demonstrate that MDMA administration produces long-term reductions in markers of the serotonergic nerve terminal (Green et al., 2003). These markers include reductions in tissue 5-HT levels, 5-HT transporter (SERT) binding, and tryptophan hydroxylase activity (Lyles and Cadet, 2003; Ricaurte et al., 2000; Sprague et al., 1998). As pointed out in a comprehensive review (O’Callaghan and Miller, 2002), evidence that these changes result from death of nerve terminals (axotomy) is based primarily on immunohistochemical analysis of 5-HT. However, the use of semiquantitative immunohistochemical methods with intact nerve terminals possessing considerably less 5-HT could produce misleading results if not supported by other neurotoxicity markers. Thus, there is clearly a need to test the hypothesis that MDMA produces axotomy using additional measures of neurotoxicity.

Several published studies examined the effect of MDMA administration on validated markers of neurotoxicity. O’Callaghan and Miller (1993, 2002) determined the effect of MDMA treatment on expression of GFAP in rats. They observed no change in GFAP expression except at exceedingly high doses (at least 75 mg/kg twice daily for 4 days). Importantly, this group reported that when MDMA and 5,7-DHT produce a similar decrease in 5-HT (about 70%), only 5,7-DHT increased GFAP (O’Callaghan and Miller, 1993). Pubill et al. (2003) administered MDMA (20 mg/kg b.i.d. × 4 days) and assessed SERT binding and glial activation 3 and 7 days posttreatment. Despite the expected decreases in SERT binding, there were no changes in several measures of glial responses. For example, MDMA administration failed to increase [3H]PK-11195 binding or induce expression of OX-6, both markers for microglial activation. Similarly, MDMA administration failed to induce astroglial activation, as indicated by Western blot measurement of GFAP and HSP27 expression. In support of these findings, Bai et al. (2001) reported that MDA administration (10 mg/kg) failed to increase GFAP expression. In contrast, Aguirre et al. (1999) reported that a single dose of MDMA (20 mg/kg, i.p.) increased GFAP expression in the hippocampus. The reasons for these discrepant results are enigmatic, but may be related to the route of administration used to administer MDMA. The other studies cited above administered MDMA via the s.c. route (Pubill et al., 2003; Bai et al., 2001; O’Callaghan and Miller, 1993, 2002), whereas Aguirre et al. (1999) administered MDMA via the i.p. route. MDMA administration to female C57BL6/J mice increased striatal GFAP expression (Miller and O’Callaghan, 1995; O’Callaghan and Miller, 1994). However, MDMA persistently depletes dopamine in the mouse, and the observed increase in GFAP expression could reflect dopaminergic axotomy. Viewed collectively, these findings indicate that MDMA-induced 5-HT depletion in the rat can occur without significant changes in GFAP expression.
Although MDMA administration fails to increase glial markers of neurotoxicity, it is well known that MDMA administration, even a single relatively low dose, results in neuronal damage as assessed by silver staining (Commins et al., 1987; Jensen et al., 1993). Similar results were obtained with a new stain, Fluoro-Jade B (Schmued and Hopkins, 2000). As reviewed in detail elsewhere (Jensen et al., 1993), much of the staining is not associated with serotonergic nerve terminals, indicating that MDMA can produce some degree of nonserotonergic brain damage. This is not surprising, since MDMA could damage nonserotonergic neurons via its hyperthermic and sympathomimetic effects (Cole and Sumnall, 2003; Green et al., 2003).

The findings reported here for the rat demonstrate that a dosing regimen of MDMA that produces long-lasting depletion of brain 5-HT fails to alter either SERT or GFAP expression. This was true even for rats that had 90% MDMA-induced 5-HT depletion. The dosing regimen we used, which was rationally chosen (see Materials and Methods), is lower than that used by some other investigators, but it produces a similar reduction in tissue 5-HT levels (Battaglia et al., 1987; Commins et al., 1987; Scanzello et al., 1993) (see Table I). In our experience, 7.5 mg/kg × 3 doses is the upper limit of dosing that can be administered without killing the rats. The protein dose–response curve reported in Figure 1 demonstrates that Western blot analysis reli-

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Fig. 3. Western blot analysis of GFAP and SERT immunoreactivity in the rat cortex, caudate, and hippocampus in MDMA treated and control groups (n = 3). Blots were digitized and quantified using densitometric analysis (NIH IMAGE software). Changes in immunoreactivity values were expressed relative to their corresponding control (defined as 100% value). Each value is the mean ± SD.
ably detects changes in SERT and GFAP expression. The decrease in SERT expression and increase in GFAP expression induced by i.c.v. administration of 5,7-DHT, a known neurotoxin, also shows that the Western blot assays can detect changes expected to occur following administration of a known neurotoxin. It is interesting that SERT expression level in 5,7-DHT-treated rats is higher than one might expect based on the degree of 5-HT depletion. A possible explanation is that, whereas 5-HT in compromised nerve terminals is likely cleared rapidly via metabolic pathways, large integral membrane proteins are likely to be cleared much more slowly. It is highly unlikely that we are measuring proteolytic fragments of the SERT, since the bands we quantitated occurred at the expected molecular weight. SERT proteins are rapidly sequestered in response to various perturbations, such as phosphorylation (Bauman et al., 2000; Blakely and Bauman, 2000; Ramamoorthy and Blakely, 1999). As with other biogenic amine transporters, SERT is subject to protein trafficking (Blakely and Bauman, 2000). Thus, SERTs in a
nerve terminal likely exist in two pools: sequestered and unsequestered. An alternative interpretation of the 5-HT “neurotoxicity” data is that MDMA produces a long-lasting shift in the balance between sequestered and unsequestered SERT so that a greater proportion of SERT is sequestered, leading to a decrease in SERT binding, but not SERT expression. The apparent loss of 5-HT nerve terminals in MDMA-treated rats can be explained by a decrease in presynaptic 5-HT to the point where it is undetectable with the immunohistochemical method, so that an apparent axotomy appears. One prediction of this alternative hypothesis is that MDMA will decrease SERT binding, or tissue 5-HT, without a corresponding decrease in SERT immunoreactive protein measured by Western blot. Whereas ligand binding methods will not detect inter-
nalized transporters, Western blot will measure all SERT protein, because denaturing conditions are used in the assay.

As noted in several reviews (Lyles and Cadet, 2003; Ricarte et al., 2000; Sprague et al., 1998), numerous variables affect MDMA responses, including dosage, species, ambient and body temperature, and route of administration (Bai et al., 2001; Esteban et al., 2001). Under the conditions used here, MDMA administration failed to produce changes consistent with axotomy. These findings are similar to those we found using d-fenfluramine and PCA (Rothman et al., 2003). The simplest explanation of our data is that, using our dosing regimen, administration of MDMA, d-fenfluramine, or PCA leads to functional inactivation of SERT, not serotonergic axotomy (Molliver and Molliver, 1990).

In conclusion, this study demonstrates that with the MDMA dosing regimen used here, MDMA does not decrease SERT expression or increase GFAP expression, a carefully validated marker of neurotoxicity (O’Callaghan and Miller, 2002). These data suggest that the putative serotonergic toxicity of substituted amphetamines should be reexamined using immunological methods to measure SERT protein changes and a wider array of neurotoxicity markers, rather than just SERT binding.

REFERENCES


