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5-HT₂ receptor-mediated potentiation of dopamine synthesis and central serotonergic deficits

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The hypothesis was tested that serotonin (5-HT) modulates 3,4-methylenedioxymethamphetamine (MDMA)-induced increase in dopamine synthesis. Rats were treated with the selective 5-HT₂ receptor agonist (R)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (R-DOI), the selective serotonin releasing agent 5-methoxy-6-methyl-2-aminoindan (MMAI), amphetamine, MDMA, or a combination of amphetamine and R-DOI or MMAI, followed by the L-dihydroxyphenylalanine (DOPA) decarboxylase inhibitor 3-hydroxybenzylhydrazine (NSD-1015). Rats were killed 45 min after the first injection and striatal DOPA was determined. R-DOI, MMAI, or amphetamine alone did not increase DOPA accumulation. However, combination of amphetamine with either MMAI or R-DOI significantly increased DOPA accumulation. Multiple doses of the R-DOI and amphetamine combination did not decrease [³H]paroxetine binding sites at one week after killing. The results indicate that the dopamine synthesis increasing effect of MDMA depends both on 5-HT₂ receptor stimulation and dopamine efflux.

Dopamine synthesis; 5-HT (5-hydroxytryptamine, serotonin); 5-HT neurotoxicity; 5-HT₂ receptors; MDMA (3,4-methylenedioxymethamphetamine); R-DOI (R)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; Amphetamine; MMAI (5-methoxy-6-methyl-2-aminoindan)

1. Introduction

The long-term effects of amphetamine derivatives such as 3,4-methylenedioxymethamphetamine (MDMA) have been intensively studied over the past five to seven years (McKenna and Peroutka 1990). It is now well established that high doses (single or multiple) of MDMA can result in depletion of serotonin (5-HT) in all major serotonin terminal fields (Stone et al., 1986; Schmidt et al., 1987; O'Hearn et al., 1988). MDMA treatment also causes long-term decreases of other serotonin terminal markers such as 5-HT uptake sites labeled by [³H]paroxetine (Battaglia, et al., 1988b; 1987; Johnson and Nichols, 1989; Nichols et al., 1990), and tryptophan hydroxylase activity (Stone et al., 1986). This persistent reduction in serotonin markers seems to correlate with the degeneration of certain serotonin axonal projections in brain (O'Hearn et al., 1988; Mamounas and Molliver, 1988; Commins et al., 1987).

Although the precise mechanism underlying these

neurotoxic effects of MDMA is not yet known, results from a number of laboratories have suggested that dopamine may play an important role in the serotonergic neurotoxicity induced by this and related compounds. For example, a positive correlation between the relative ability of MDMA-like drugs to induce dopamine release in vitro and their ability to induce a neurotoxic response in vivo has been previously noted (e.g. Johnson et al., 1990; Nichols et al., 1990; Schmidt et al., 1987). Agents which interfere with the dopamine release induced by MDMA can prevent or attenuate its toxic effects on the serotonergic system. For example, the dopamine uptake inhibitor GBR-12909 attenuates the MDMA-induced persistent changes in serotonergic markers (Stone et al., 1988). Depleting dopamine stores with α -methyl-p-tyrosine attenuates the long-term serotonergic effects of MDMA in the striatum, hippocampus and cortex (Stone et al., 1988; Schmidt et al., 1990). Similarly, decreasing catecholamine stores either with reserpine (Stone et al., 1988; Schmidt et al., 1990) or with the decarboxylase inhibitor monofluoromethyl-DOPA (Schmidt et al., 1990) attenuates or blocks the persistent serotonergic deficits induced by MDMA. Furthermore, selectively induced lesions of the nigrostriatal dopaminergic system with bilateral injections of 6-hydroxydopamine in the substantia nigra block the

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long-term serotonergic effects of MDMA (Stone et al., 1988; Schmidt et al., 1990).

Other studies, particularly from our laboratory have suggested that 5-HT release is a necessary but not sufficient component of the 5-HT neurotoxicity process induced by MDMA. The relatively 'pure' and potent 5-HT releaser 5-methoxy-6-methyl-2-aminoindan (MMAI) (Johnson et al., 1991) will cause long term central 5-HT deficits only when combined with amphetamine, while neither drug alone induced 5-HT neuron toxicity (Johnson and Nichols, 1991; Johnson, 1991). Agents which interfere with 5-HT release also have been shown to block serotonergic neuron toxicity. For example, 5-HT uptake blockers such as fluoxetine can block MDMA-induced long-term 5-HT deficits (Schmidt et al., 1987).

Curiously, selective 5-HT₂ receptor antagonists such as ketanserin and MDL 100,907 can also prevent the long-term neurotoxic effects of MDMA (Nash, 1990; Schmidt and Kehne, 1990 and Schmidt et al., 1992).

The studies by Nash et al. (1990) originally indicated that the protective effect of 5-HT₂ receptor antagonists may occur via alteration in the dopaminergic system. Those studies showed that ketanserin could block both the acute stimulation of dopamine synthesis produced by MDMA, as well as the serotonergic deficits measured one week later. A subsequent study demonstrated that ketanserin could also attenuate the *in vivo* release of dopamine induced by high doses of MDMA (Nash, 1990). The link between this acute effect of the 5-HT₂ receptor antagonists on dopamine synthesis and their ability to prevent the long-term neurochemical effects of MDMA is further strengthened by experiments showing that the protective effect of the 5-HT₂ receptor antagonist MDL 11,939 was blocked if dopamine synthesis was restored by the administration of L-dihydroxyphenylalanine (DOPA) (Schmidt et al., 1991). The above studies lead to the question: does 5-HT released by MDMA play a role in serotonin neuron toxicity by increasing dopamine synthesis through activation of 5-HT₂ receptors? Also, we were interested to learn whether increasing dopamine synthesis and release coupled with concomitant 5-HT₂ receptor stimulation would be sufficient to induce long term serotonergic deficits.

In the present studies, the 5-HT₂ receptor agonist (R)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (R-DOI; Glennon, 1987) and the non-vesicular dopamine releasing agent amphetamine were employed to investigate further the role of 5-HT₂ receptor stimulation and non-vesicular dopamine release in MDMA-induced neurotoxicity. The lowest dose of DOI was chosen that would be expected, with certainty, to produce a biochemical effect. Dimpfel et al. (1989) showed that 0.1 mg/kg of DOI produced significant changes in the rat EEG. However, Paulus and Geyer

(1992), using a computerized behavioral pattern monitor, did not detect behavior changes in rats with an acute dose of 0.27 mg/kg, but did observe marked effects with a dose of 0.81 mg/kg. Recently, in our laboratory, a dose of 0.4 mg/kg was found to be the lowest dose that could be used to train rats to reliably discriminate DOI from saline in a drug discrimination paradigm (unpublished data). These results were considered in the selection of the 0.5 mg/kg dose of DOI in the present studies.

2. Materials and methods

2.1. Materials

(+)-Amphetamine sulfate was purchased from Smith Kline and French Laboratories (Philadelphia, PA). MDMA and MMAI hydrochlorides were synthesized in our laboratory (Nichols et al., 1986; Johnson et al., 1991). R-DOI hydrochloride was synthesized in our laboratory by standard methods. [³H]Paroxetine was purchased from New England Nuclear (Boston, MA) at a specific activity of 20.5 Ci/mmol. The HPLC standards and 3-hydroxybenzylhydrazine dihydrochloride (NSD 1015) were purchased from Sigma Chemical Company (St. Louis, MO). All drugs injected into animals were dissolved in saline and administered in a volume of 1 ml/kg. NSD 1015 was injected intraperitoneally (i.p.); other drugs were administered subcutaneously (s.c.).

2.2. Animals

Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 175–200 g were used in all experiments. Animals were individually caged in a temperature controlled room with a 12/12 h lighting schedule. Food and water were available *ad libitum* at all times. Animals were killed by decapitation, and the brain was rapidly removed. Frontal cortex, hippocampus and striatum were dissected out over ice. Each tissue was individually wrapped and kept at –70°C until assay.

2.3. DOPA accumulation in rat striatum

Measurement of DOPA accumulation after different drugs and drug combinations basically followed the procedure of Nash et al. (1990). Briefly, DOPA accumulation in the striatum was determined 45 min after the s.c. administration of 0.5 mg/kg R-DOI, 20 mg/kg MMAI, 2.5 mg/kg amphetamine, 20 mg/kg MDMA, or a combination of 2.5 mg/kg amphetamine with 0.5 mg/kg R-DOI or with 20 mg/kg MMAI. NSD 1015 (100 mg/kg) was given 30 min prior to decapitation.

High pressure liquid chromatography (HPLC) with electrochemical detection (EC) was used to determine biogenic amines and metabolite levels. A mobile phase containing 50 mM NaH_2PO_4 , 30 mM citric acid, 0.1 mM Na_2EDTA , 0.034% sodium octyl sulfate and 23–25% v/v methanol was used. The brain samples were prepared by homogenizing the weighed brain areas from one hemisphere in 0.5 ml of HPLC mobile phase without sodium octyl sulfate, using a motor-driven Teflon pestle and Eppendorf 1.5 ml centrifuge tube. The samples were then centrifuged at $14,000 \times g$ for 5 min with a table top centrifuge. The supernatant was assayed for monoamines and their metabolites by injection of 50 μl onto a Brownlee C18 analytical cartridge column (Ansco, Ann Arbor, MI) with a flow rate of 0.7 ml/min. The HPLC-EC system consisted of a refrigerated autosampler (TosoHaas, Philadelphia, PA), and a model 400 EG&C Princeton electrochemical detector (EG and G PARC, Princeton, NJ) with a dual electrode potential set at $E_1 = -200$ mV and $E_2 = 850$ mV versus the Ag/AgCl reference electrode.

2.4. [^3H]Paroxetine binding 1 week after treatments

Animals were given single or multiple (twice a day for 4 days) doses of 0.5 mg/kg R-DOI, 2.5 mg/kg amphetamine, 20 mg/kg MDMA, or a combination of 0.5 mg/kg R-DOI with 2.5 mg/kg amphetamine. One week later animals were killed as described under section 2.2.

A modified procedure of Marcusson et al. (1988) was employed to measure [^3H]paroxetine binding sites. A buffer consisting of 50 mM Tris HCl with 120 mM NaCl and 5 mM KCl at pH 7.4 was utilized both for incubations and for tissue homogenization. Tissue samples were weighed and homogenized in 5 ml of the buffer described above with a Brinkman polytron (setting 6, 2×20 s). The homogenates were centrifuged twice at $30,000 \times g$ for 10 min with an intermittent wash and the pellet was resuspended in the same volume of the buffer described above.

Since it has previously been reported that only the B_{max} and not the K_D values are altered after MDMA treatment (Battaglia et al., 1987; Nichols et al., 1990), it is possible to estimate the number of 5-HT uptake sites with a single saturating (1 nM) concentration of [^3H]paroxetine (Battaglia et al., 1988b; Johnson et al., 1990). Nonspecific binding was defined with 1 μM fluoxetine. Incubations were started by adding 150 μl of tissue homogenate to each tube to give a final volume of 1.65 ml. Tubes were allowed to equilibrate at 24°C for 1 h before adding 4 ml of ice cold buffer and filtering through Whatman GF/C filters using a Brandel Cell Harvester (Gaithersburg, MD). The tubes were washed twice with ice cold buffer and the filters allowed to air dry. Filters were then placed into 20 ml

plastic vials and 10 ml of scintillation counting fluid was added. The vials were sealed and allowed to sit overnight before counting at an efficiency of 37%.

2.5. Statistical analysis

Values are the means $\pm$ S.E.M. for eight animals in each treatment group. Separate saline controls were run in parallel with each experiment. All comparisons utilized an analysis of variance (ANOVA) followed by a modified t-test post hoc comparison as embodied in the computer program EPISTAT (EPISTAT Services, Richardson, TX).

3. Results

Consistent with the results of Nash et al. (1990), MDMA significantly increased the DOPA level in rat striatum (fig. 1A). As shown in fig. 1B, 0.5 mg/kg R-DOI, or 20 mg/kg MMAI did not change the DOPA level significantly. Tyler and Galloway (1992) have previously reported that amphetamine (3 mg/kg) signifi-

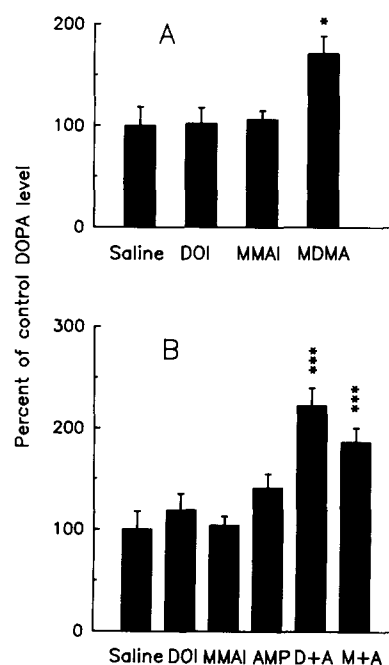


Fig. 1. The effects of different drugs and drug combinations on DOPA accumulation in the rat striatum. Saline, R-DOI (DOI) 0.5 mg/kg, amphetamine (AMP) 2.5 mg/kg, MMAI 20 mg/kg, MDMA 20 mg/kg, or a combination of AMP 2.5 mg/kg with R-DOI 0.5 mg/kg (D + A) or with MMAI 20 mg/kg (M + A) was injected s.c. 45 min prior to killing and 15 min before i.p. injection of 100 mg/kg NSD 1015. The DOPA level was determined in striatum using HPLC-EC techniques. Each value represents the mean $\pm$ S.E.M. for $n = 8$. The saline control values were 831 ± 155 and 1227 ± 221 pg/mg wet weight for panel A and B, respectively. * Indicates significantly different from saline control value ($P < 0.01$, ANOVA). *** Indicates significantly different from saline control value ($P < 0.0001$, ANOVA).

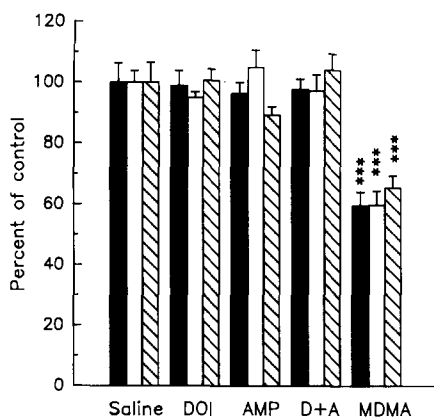


Fig. 2. [^3H]Paroxetine binding one week after multiple doses of drugs and drug combinations. Saline, R-DOI (DOI) 0.5 mg/kg, amphetamine (AMP) 2.5 mg/kg, MDMA 20 mg/kg, or a combination of R-DOI 0.5 mg/kg and amphetamine 2.5 mg/kg (D+A) was injected s.c. This was repeated every 12 h for 4 days. Animals were killed 1 week after the last dose. The number of serotonin uptake sites was determined using [^3H]paroxetine binding following a modified procedure of Marcusson et al. (1988) as described in Methods. Values are presented as the means $\pm$ S.E.M. for $n = 8$. Saline control values were as follows with units of fmol/g wet weight: cortical (black columns), 19.5 ± 1.2 ; hippocampus (open columns), 17.8 ± 0.7 ; striatum (hatched columns), 26.3 ± 1.7 . *** Indicates significantly different from saline control value ($P < 0.0001$, ANOVA).

cantly increases DOPA accumulation. However, in the present experiment, although 2.5 mg/kg amphetamine appears to increase DOPA, this effect did not reach significance ($P = 0.062$). The combination of 2.5 mg/kg amphetamine with 0.5 mg/kg R-DOI or with 20 mg/kg MMAI also significantly increased the DOPA level ($P < 0.0001$).

Treatment with a combination of MMAI and amphetamine has been shown to induce significant deficits in 5-HT neuronal markers in rats (Johnson and Nichols, 1991) at doses where neither drug alone had long term effects. The ability of the R-DOI and amphetamine combination to induce a similar neurotoxicity was assessed in the present study with MDMA treatment serving as a parallel positive control. Neither single (data not shown) nor multiple doses of the R-DOI and amphetamine combination decreased [^3H]paroxetine binding sites at one week after killing, while parallel MDMA treatment significantly decreased this parameter by almost half (fig. 2).

4. Discussion

A recent study by Schmidt et al. (1992) suggested that 5-HT $_2$ receptors exert a 'state-dependent' regulation of dopaminergic function. In their studies, the 5-HT $_2$ receptor antagonist MDL 100,907 attenuated the MDMA-induced increase in extracellular concentration of striatal dopamine and the increased dopa-

mine synthesis while having no effect on basal, haloperidol, or reserpine stimulated dopamine synthesis. They suggested that the regulation of dopamine synthesis mediated by 5-HT $_2$ receptors is likely to be a phasic rather than a tonic effect which becomes significant only during states of high serotonergic and dopaminergic transmission. The results of our present studies are consistent with this hypothesis by showing that 5-HT $_2$ receptor stimulation or dopamine release alone could not increase dopamine synthesis. However, a combination of both these events significantly increased dopamine synthesis (fig. 1). This result more clearly shows that both 5-HT $_2$ receptor stimulation and nonvesicular dopamine release are needed to produce large increases in dopamine biosynthesis.

The increase of dopamine synthesis and release has been implied to play a very important role in MDMA induced neurotoxicity (e.g. Stone et al., 1988; Schmidt et al., 1990 and 1991; Johnson et al., 1991). The present studies show that increasing dopamine synthesis by combined administration of a 5-HT $_2$ receptor agonist and nonvesicular dopamine releaser are not sufficient to produce a long-term serotonin deficit, measured by [^3H]paroxetine binding experiments.

However, combination of the nonvesicular serotonin releaser MMAI with the nonvesicular dopamine releaser amphetamine did induce serotonergic deficits (Johnson and Nichols, 1991). A similar (or even higher) degree of stimulation of dopamine synthesis (as shown in fig. 1) was obtained with either the MMAI and amphetamine combination or the R-DOI and amphetamine combination treatment, compared with MDMA.

In view of the fact that MMAI lacks direct 5-HT $_2$ receptor agonist action (Nichols et al., 1993) one must infer that 5-HT $_2$ receptor stimulation occurs as a consequence of the released endogenous 5-HT. Although MDMA does have weak direct 5-HT $_2$ receptor agonist properties, (+)-MDMA is the more neurotoxic isomer, while it is the (–)-isomer that has higher 5-HT $_2$ receptor affinity (Lyon et al., 1986; Battaglia et al., 1988a and Johnson et al., 1988). This suggests that the 5-HT $_2$ receptor agonist effects of MDMA are also chiefly attributable to the stimulation of 5-HT $_2$ receptors by released endogenous 5-HT.

It is suggested by these results that factors other than dopamine synthesis and release are also necessary for the neurotoxicity induced by MDMA. Thus, one may envisage at least one scenario which leads to 5-HT neurotoxicity. The release of nonvesicular dopamine, coupled with the stimulation of 5-HT $_2$ receptors by the concomitant release of neuronal 5-HT leads to a marked increase both of dopamine synthesis and consequent dopamine efflux from the terminal. At the same time, neuronal 5-HT release appears to be necessary since the direct stimulation of 5-HT $_2$ receptors by

R-DOI did not lead to neurotoxicity. The question of whether 5-HT depletion from the neuron terminal then renders it vulnerable to dopamine toxicity or the action of some other toxin needs to be addressed by further studies.

In conclusion, the dopamine synthesis-increasing effect of MDMA depends on its ability both to stimulate the 5-HT₂ receptor, and to induce dopamine efflux; increased dopamine synthesis and efflux alone are not sufficient to explain the serotonergic neurotoxic effects induced by MDMA.

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