# MDMA (Ecstasy) effects on cultured serotonergic neurons: evidence for $Ca^{2+}$-dependent toxicity linked to release

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Animal studies have established a correlation between release of 5-hydroxytryptamine (5-HT) and the long-term reduction of 5-HT (toxicity) by 3,4-methylenedioxymethamphetamine (MDMA) with the S(+) enantiomer being more active than the R(−). Using a microculture system of fetal raphe neurons, the enantiomers of MDMA were tested to determine if a similar difference in potency existed. The results showed that the development of the uptake capacity of [$^3$H]5-HT in 4-day cultures was half-maximally inhibited by a single application at time of plating of $5 \times 10^{-6}$ M S(+)-MDMA and $5 \times 10^{-5}$ M R(−)-MDMA. In order to determine if the $Ca^{2+}$-independent release (chemically induced through the transporter protein and inhibited by reuptake blockers) or the $Ca^{2+}$-dependent release ($K^+$-induced and inhibited by presynaptic receptors) contributed to the toxicity, fluoxetine and $D_1$ and $\alpha_2$ agonists were studied. The results showed that both forms of release were involved in the loss of [$^3$H]5-HT uptake capacity, with the direct MDMA-induced $Ca^{2+}$-independent (fluoxetine-sensitive) release being the first step. Evidence from binding studies indicates that MDMA has a micromolar affinity for the 5-$HT_2$ receptor, and our studies in culture showed that ketanserin, a specific 5-$HT_2$ antagonist, was effective at attenuating the effects of S(+)-MDMA on the development of the [$^3$H]5-HT uptake capacity by the cultured raphe neurons. The 5-$HT_2$ receptor is linked to increased intracellular $Ca^{2+}$ through a second messenger phosphatidylinositol (PI)-hydrolysis mechanism. Since increases in $Ca^{2+}$ intracellular levels have been shown to be a key step in neuronal death, the interaction between MDMA and caffeine (stimulates release of $Ca^{2+}$ from intracellular stores) and nimodipine (a $Ca^{2+}$ channel blocker) were studied. The results showed that caffeine ($10^{-6}$ M) potentiated and nimodipine ($10^{-9}$ M) attenuated the inhibitory effects of MDMA. Our results are consistent with both types of 5-HT release being involved in producing a $Ca^{2+}$-dependent toxicity induced by MDMA on cultured serotonergic fetal neurons.

## INTRODUCTION

A number of drugs whose primary mode of action is the $Ca^{2+}$-independent release of monoamines produce permanent or long-lasting changes in brain monoamine levels. For example, parachloramphetamine, methamphetamine, and fenfluramine administered to rats depletes serotonin and dopamine levels for weeks to months[17,22,32,39,42,46]. This property is shared with the hallucinogenic amphetamines whose parent compound is 3,4-methylenedioxyamphetamine (MDA). These drugs, which include 3,4-methylenedioxymethamphetamine (MDMA) and (3,4-methylenedioxyethamphetamine (MDE) also cause a release of serotonin and produce long-lasting changes in brain 5-hydroxytryptamine (5-HT) levels in rats and monkeys[40,41,43,51]. The decrease in monoamine levels probably reflects neurodegeneration of the neurons in the brain. In fact, immunocytochemical studies have established that a major portion of the serotonergic axons in the cortex and hippocampus are lost after MDMA and PCA injections[35,53].

Nichols and colleagues[34] have proposed that the $Ca^{2+}$-independent transmitter release was related to the psychotrophic properties of MDMA since the isomers of MDMA differ both in their ability to release 5-HT in vitro and in their psychotrophic effectiveness. Schmidt and co-workers[45] reported that the S(+)-MDMA, the more psychoactive enantiomer, is also more effective at producing the long-term neurodegenerative changes. The $Ca^{2+}$-independent release by MDMA has been shown to be inhibited by specific reuptake blockers[4,47]. The co-administration of these blockers in vivo can prevent the long-term decrease in 5-HT levels produced by MDMA and related drugs[5]. Therefore, we used our tissue culture of dissociated fetal serotonergic neurons to compare the effects of the enantiomers of MDMA and to test the

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protective effects of the potent 5-HT reuptake blocker, fluoxetine[16].

The $Ca^{2+}$-dependent release of monoamines is generally assumed not to be involved in the actions of MDMA or other related amphetamine drugs. This vesicular release of serotonin from presynaptic terminals by a $K^+$-induced depolarization-mediated process is under the negative control of a number of presynaptic receptors including the dopaminergic $D_1$[6] and the adrenergic $\alpha_2$[14,25,38]. Thus, if the toxicity is independent of the $Ca^{2+}$-dependent release of 5-HT the addition of one of the presynaptic agonists known to inhibit release of 5-HT should have no effect upon the toxicity. We studied the effects of the SKF-38393 for $D_1$[6] and the BH-T 920 for the $\alpha_2$ receptor[36] on the effects of MDMA on cultured serotonergic fetal neurons.

The final problem we investigated concerns the mechanism by which release might actually produce the toxicity. In order to test if excess $Ca^{2+}$ influx contributes to the toxicity produced by MDMA on cultured serotonergic neurons, the effects of a 1,4-dihydropyridine (DHP) $Ca^{2+}$ channel blocker, nimodipine[20], was studied. This $Ca^{2+}$ channel antagonist closes a $Ca^{2+}$ channel but does not directly affect transmitter uptake or release when used at low concentrations[8,23]. Therefore, any protective effect should be related to net $Ca^{2+}$ flow rather than to inhibition of release.

A related question deals with the intracellular release of stored $Ca^{2+}$. It has been reported that MDMA binds to a 5-$HT_2$ receptor[4,19]. This receptor is linked to a PI second messenger system[12]. One result of increased PI production would be the increased release of intracellular $Ca^{2+}$ from storage sites[7]. We studied the effects of ketanserine, a specific 5-$HT_2$ antagonist[28] to test if this drug attenuated the MDMA toxicity. If the toxicity was related to $Ca^{2+}$ increase via this mechanism, then addition of caffeine, which also releases intracellular $Ca^{2+}$ (ref. 52), should potentiate the MDMA-induced toxicity.

The results indicate that the inhibitory action of MDMA are greater for the S(+) enantiomer and can be attenuated by fluoxetine, both $D_1$ and $\alpha_2$ presynaptic agonists, the 5-$HT_2$ antagonist and a calcium channel antagonist and potentiated by caffeine. The findings are consistent with the hypothesis that MDMA toxicity is linked to both a cytoplasmic ($Ca^{2+}$-independent) and vesicular ($Ca^{2+}$-dependent) release of 5-HT and suggest that the toxicity may be related to an intracellular $Ca^{2+}$ buildup from both storage (5-$HT_2$-sensitive) and extracellular pools of $Ca^{2+}$ (DHP-sensitive).

## MATERIALS AND METHODS

We obtained maximum-barrier (pathogen-free), timed pregnant rats (14 dg) from Taconic Farms (Pennsylvania). The fetuses were removed by Caesarian section using antiseptic procedures, and transferred in ice-cold D1-solution containing magnesium and calcium-free minimal essential medium (MEM) and the antibiotics penicillin and streptomycin. A rostral rhombencephalon and caudal mesencephalon tegmental slice was dissected which contained the majority of the midbrain serotonergic cell bodies[3]. The underlying meninges were removed and the slice kept in cold D1 solution. The slices were transferred to 0.5 ml of D1 solution, minced and gently agitated by repeated trituration using a Pasteur pipette with a fire-polished tip. The cell suspension was then brought up to 1.5 ml/fetus with complete neuronal media (CNM) and allowed to settle. The CNM consisted of MEM with non-essential amino acids (180 ml), fetal calf serum (15 ml, Gibco), and 0.5% glucose supplement (MEM contains 0.1% glucose).

The 14-day-old fetal cells were plated onto 96-well plates (Nunc, Denmark) previously coated with poly-D-lysine (15 $\mu$g/ml, 70,000 MW, Sigma). The poly-D-lysine was added the night before and removed before use. The cells were plated at an initial plating density (IPD) of approximately $0.9 \times 10^6$ cells/cm$^2$ by adding 200 $\mu$l of the cell suspension to each well (area of 0.28 mm$^2$).

Cultures were rinsed before incubation with fresh HBSS containing 0.6% glucose and the radioactive neurotransmitter [$^3$H]5-HT (50 nM, New England Nuclear, spec.act. 15–27 Ci/mmol) for 20 min at 37 °C. Non-specific uptake was determined in the presence of 100 nM fluoxetine and averaged about 10% of the total uptake[2]. At the end of a 20-min period, the incubation media was removed, and the cultures were washed twice with 100 mM PBS. The cells were allowed to air dry before adding 200 $\mu$l of absolute ethanol for one hour to remove the [$^3$H]5-HT retained by the cells. From each well 150 $\mu$l of ethanol are removed and added to 5 ml of Liquiscent (National Diagnostics, Manville, NJ) and counted for at least 1 min in a Beckman liquid scintillation counter (Model 1801; counting efficiency is 58%).

S(+) and R(−) MDMA (NIDA, Washington, DC) were prepared in serial dilution and applied at time zero in culture. Fluoxetine (Eli Lilly Co., Indianapolis, IN), BH-T 920 (Boehringer-Mannheim Co., West Germany), SKF-38393 (Smith-Kline & French, Philadelphia, PA), ketanserine tartrate (Research Biochemical Incorp., Natick, MA) caffeine (Sigma Chemical Co., St. Louis, MO), and nimodipine (Miles Research Lab, New Haven, CT) were administered at the same time as MDMA. All drug solutions were prepared in sterile MEM and passed through Millipore filters (0.2 $\mu$m, Millipore Co.). Preliminary studies were performed on serial dilutions of all the drugs used to determine the concentration range in which they were the most active in our tissue culture system.

Statistics were performed with a Systat software program (Systat, Inc., Evanston, IL) using ANOVA and Tukey post-hoc test. Individual significance tests were also run using Student's $t$-test. Graphs were generated on Sigma Plot software program (Jandel Scientific Co., Sausalito, CA). All experiments reported were replicated at least once.

## RESULTS

Fetal serotonergic neurons dissociated from the rat mesencephalic raphe area survive in microculture for up to 5 days without a media change[2]. The uptake capacity for [$^3$H]5-HT ($5 \times 10^{-8}$ M) by serotonergic neurons increases over time and is correlated to neuronal maturation. The effects of the two enantiomers of MDMA [S(+) and R(−)] after a single application at time zero were studied on the development of the uptake capacity after 3 days in culture (Fig. 1). It can be seen that the S-MDMA isomer produced a half-maximal inhibition at

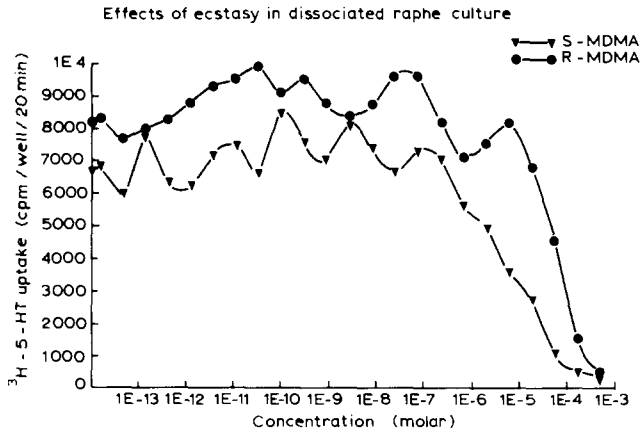


Fig. 1. The effects of the enantiomers of MDMA, S(+) and R(−), were tested on the development of the [$^3$H]5-HT uptake capacity by fetal serotonergic neurons after 3 days in culture. The drugs were applied at the time of initial plating. Each point represents the mean of 4 cultures. The half maximal inhibition with S-MDMA was $5 \times 10^{-6}$ M and with R-MDMA $5 \times 10^{-5}$ M. The analysis of variance showed significance for both S-MDMA ($F = 62.8$, df = 23, $P < 0.0001$, post-hoc Tukey for $P < 0.05$ gives critical range for pairs of means = 1630) and R-MDMA ($F = 18.4$, df = 23, $P < 0.0001$, post-hoc Tukey for $P < 0.05$ gives critical range for pairs of means = 2832)

a concentration of $5 \times 10^{-6}$ M, while the R-MDMA isomer reached a half-maximal inhibition at $5 \times 10^{-5}$ M. This decrease was found to proceed in a dose-related manner.

The in vivo long-term changes seen in 5-HT levels after MDMA administration are prevented by co-administration of a 5-HT reuptake blocker. In our culture experiments, the addition of fluoxetine ($10^{-7}$ M) prevented the toxicity and stimulation seen with the S(+)-MDMA at $10^{-4}$ and $10^{-10}$ M, respectively (Table I). The inhibition seen with $10^{-4}$ M R(−) was less than seen with the S(+) MDMA, and fluoxetine ($10^{-7}$ M) non-significantly reduced this inhibition. Fluoxetine ($10^{-5}$ M) produced a strong inhibition by itself (35% of control) and this was not affected by co-administration of MDMA. These results are consistent with the studies in vivo and suggest that the $Ca^{2+}$-independent release blocked by an uptake inhibitor is the initial event in the MDMA mediated neurotoxicity in serotonergic neurons.

The action of ketanserine ($10^{-8}$ M and $10^{-6}$ M) on the effects of the enantiomers of MDMA ($10^{-10}$ M and $10^{-4}$ M) are shown in Table I. The 5-$HT_2$ antagonist at $10^{-8}$ M was effective at reversing the effects of S(+) MDMA. The effects on R(−) MDMA inhibition and with $10^{-6}$ M ketanserine were in the same direction but did not reach significance with the Tukey post-hoc analysis test (Table I).

An increase in $Ca^{2+}$ release from an intracelluar pool may contribute to the toxicity, as might be predicted by the finding that MDMA binds to a 5-$HT_2$ receptor. This question was addressed by examining whether caffeine which potentiates the release of $Ca^{2+}$ from intracellular sources, would also potentiate the toxicity of S(+)-MDMA. Caffeine ($10^{-6}$ M) had no direct effect on the cultured 5-HT neurons after 3 days of incubation (Fig. 2). S-MDMA at $10^{-6}$ M produced a significant reduction of the development of the uptake capacity in the cultured serotonergic neurons. Co-administration of caffeine was able to cause a significant inhibition compared to control cultures with $10^{-6}$ M of S-MDMA and the inhibition at $10^{-6}$ M was significantly lower than seen with S-MDMA alone (Fig. 2).

In order to determine if the $Ca^{2+}$-dependent release played any role in the MDMA action, we studied the effects of receptor agonist which inhibit this release. The alpha agonist BH-T 920 acts on serotonergic presynaptic axons to inhibit the $K^+$-induced release of 5-HT. BH-T 920, at a concentration of $1 \times 10^{-9}$ M produced no change in [$^3$H]5-HT uptake capacity in the control cultures (3028 ± 250 versus 3659 ± 560, mean ± S.E.M., $n = 4$, control and BH-T 920 respectively). The inhibition produced by $10^{-5}$ M S(+)MDMA and R(−)MDMA (458 ± 59 and 1403 ± 86, respectively) was significantly attenuated in

TABLE I

*Effects of fluoxetine and ketanserine on MDMA action in culture*

All drugs were added at time zero and the cultures grown for 3 days before performing the high-affinity uptake with [$^3$H]5-HT (50 nM). The results are shown as the mean ± S.E.M. ($n$). Analysis of variance $F = 9.769$, df = 23; $P < 0.0001$. Tukey post-hoc statistical test to their respective controls: $^*P < 0.05$; $^{**}P < 0.01$ and ▲ = $P < 0.05$; ▲▲ = $P < 0.01$.

| *Treatment* | *Control* | *Fluoxetine* | | *Ketanserine* | |
|---|---|---|---|---|---|
| | | *$10^{-7}$ M* | *$10^{-5}$ M* | *$10^{-8}$ M* | *$10^{-6}$ M* |
| Control | 773 ± 66 (2) | 723 ± 86 (4) | 289 ± 57 (3) | 829 ± 56 (3) | 816 ± 52 (3) |
| S-MDMA ($10^{-10}$ M) | 1526 ± 100 (3)▲▲ | 926 ± 66 (3)* | 320 ± 71 (4)** | 840 ± 86 (3)** | 1110 ± 147 (4) |
| S-MDMA ($10^{-4}$ M) | 184 ± 44 (3)▲ | 969 ± 40 (3)** | 384 ± 148 (4) | 877 ± 85 (3)** | 508 ± 168 (4) |
| R-MDMA ($10^{-10}$ M) | 678 ± 19 (3) | 1031 ± 40 (4) | 464 ± 135 (3) | 657 ± 85 (4) | 853 ± 130 (3) |
| R-MDMA ($10^{-4}$ M) | 268 ± 68 (4)▲ | 661 ± 84 (3) | – | 615 ± 183 (3) | 715 ± 43 (3) |

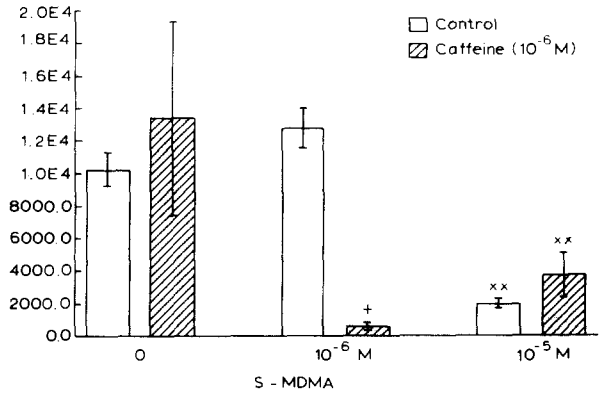


Fig. 2. The effects of caffeine ($10^{-6}$ M) on the loss of [$^3$H]5-HT uptake capacity in cultured serotonergic neurons following a single application of MDMA ($10^{-6}$ M). All drugs added at the time of initial plating. The bars represent the average ± S.E.M. of 4 cultures. The analysis of variance showed significance ($F = 19.0$, df = 6, $P < 0.0001$). Student's $t$-test: $^{+}P < 0.01$; $^{++}P < 0.05$ with respect to MDMA ($10^{-6}$ M) alone.

the presence of BH-T 920 (1707 ± 259 and 1788 ± 98; $P < 0.01$ and $P < 0.05$, respectively, Student's $t$-test).

In another experiment, a $D_1$ agonist (SKF-38393) was used in an analogous fashion to the $\alpha_2$ agonist. SKF-38393 has also been shown to block 5-HT presynaptic $K^+$-induced release but through the $D_1$ dopamine receptor. The two release blockers were tested against the inhibition caused by S-MDMA at $10^{-5}$ M and $10^{-6}$ M (40 and 70% of control, $P < 0.01$). The administration of the $\alpha_2$ or the $D_1$ agonist significantly attenuated the inhibition with $10^{-5}$ M MDMA and completely reversed the inhibition seen at $10^{-6}$ M (Fig. 3).

To determine if the MDMA toxicity might be related to an influx of extracelluar $Ca^{2+}$, a 1,4-dihydropyridine

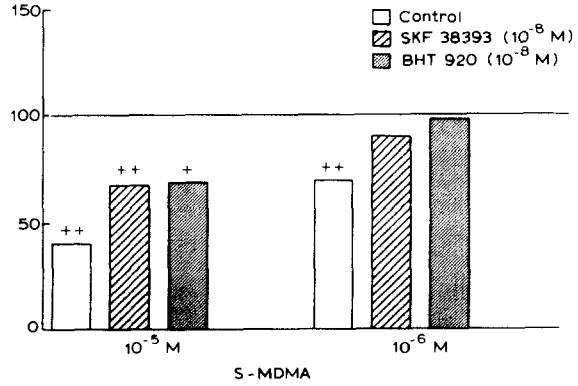


Fig. 3. The effects of an $\alpha_2$ (BH-T 920) and $D_1$ (SKF 38393) receptor agonists at $10^{-8}$ M on the effects of S-MDMA ($10^{-5}$ and $10^{-6}$ M). All drugs were applied at the time of initial plating. The results are expressed as a percentage of the control value (13,5318 ± 873; average ± S.E.M., $n = 4$). Each bar represents the results of 4 cultures. Analysis of variance performed on the raw data ($F = 11.1$, df = 8, $P < 0.0001$). Student's $t$-test with respect to control: $^{++}P < 0.01$; $^{+}P < 0.05$, and with respect to MDMA value, indicated by second row.

$Ca^{2+}$ channel blocker was tested in culture. Previous studies had shown that $1 \times 10^{-9}$ M nimodipine could prevent the toxic effects of high external $Ca^{2+}$ in our culture medium. This concentration of nimodipine was co-administered with either S-MDMA or R-MDMA at 5 concentrations ($10^{-6}$ M, $5 \times 10^{-6}$ M, $10^{-5}$ M, $5 \times 10^{-5}$ M and $10^{-4}$ M). Nimodipine was effective at preventing the inhibition seen when the MDMA enantiomers were added alone (Fig. 4).

A proposed model of neuronal toxicity of serotonergic neurons related to MDMA action involves at least 4 separate steps (Fig. 5). Step 1: MDMA reacts with the transporter to release cytoplasmic 5-HT and inhibit 5-HT reuptake (blocked by fluoxetine). Step 2: MDMA reacts with the 5-$HT_2$ receptor to stimulate PI-hydrolysis and release intracellular $Ca^{2+}$ (attenuated by ketanserine and potentiated by caffeine). Step 3: increased intracelluar $Ca^{2+}$ causes release of vesicular 5-HT (blocked by presynaptic $D_1$ and $\alpha_2$ agonist). Step 4: influx of extracelluar $Ca^{2+}$ in response to vesicular release (blocked by nimodipine).

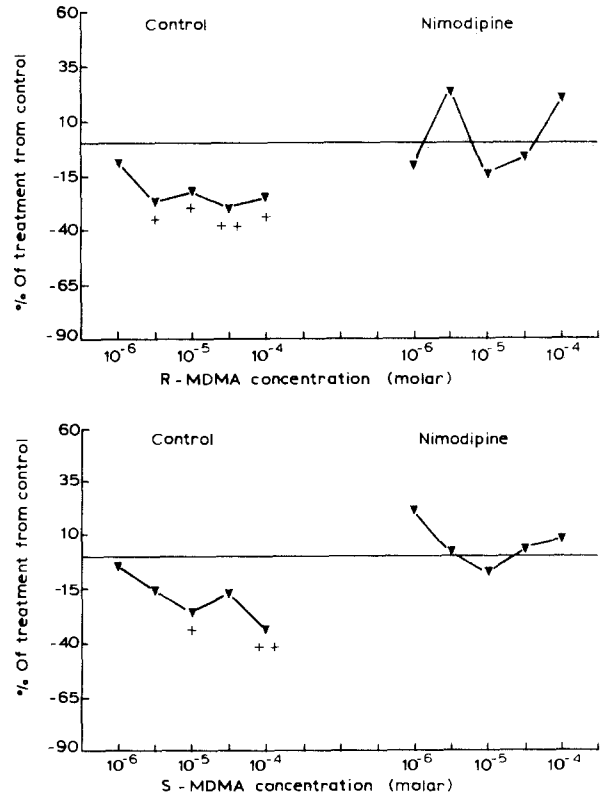


Fig. 4. The effects of nimodipine ($10^{-9}$ M) on the inhibition produced by S-MDMA (top panel) and R-MDMA (bottom panel). All drugs were added at the time of initial plating and expressed as percentage of their respective control values (control = 497 ± 28 and 478 ± 26; nimodipine = 1762 ± 131 and 1970 ± 93; with respect to top and bottom panels). Student's $t$-test was performed on raw data with respect to control or nimodipine alone values: $^{+}P < 0.05$; $^{++}P < 0.01$.

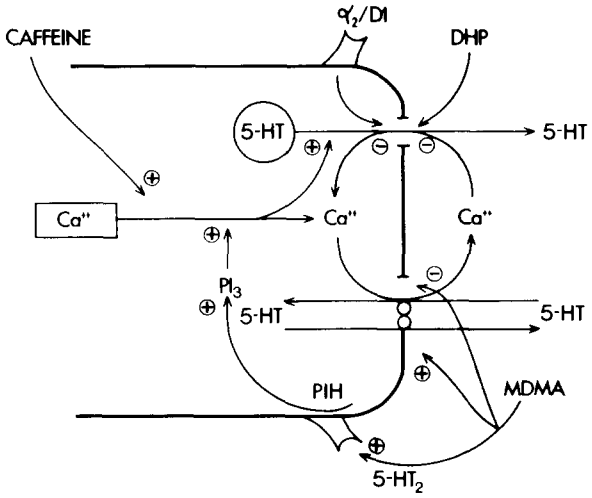


Fig. 5. A diagramatic summary of 5-HT terminals showing two types of serotonin release: a $Ca^{2+}$-independent cytoplasmic release mediated by a transporter mechanism and a $Ca^{2+}$-dependent vesicular release. MDMA stimulates the cytoplasmic release and inhibits the reuptake of 5-HT which is $Ca^{2+}$-dependent. Fluoxetine is believed to displace MDMA from both these sites and prevents both the release and the toxicity. MDMA also interacts with the 5-$HT_2$ receptor which stimulates the hydrolysis of phosphatidylinositol (PIH) to produce inositol 1,4,5-triphosphate ($PI_3$) and release stored $Ca^{2+}$. Ketanserine, a 5-$HT_2$ receptor anatagonist, can attenuate the toxicity while caffeine potentiates the toxicity. Our model predicts that the increase in intracellular $Ca^{2+}$ would activate the release of vesicular 5-HT which would in turn produce an increase influx of $Ca^{2+}$. This $Ca^{2+}$-dependent release is blocked by the presynaptic $\alpha_2$ and $D_1$ receptor agonists. Nimodipine, a 1,4-dihydropyridine (DHP) is a $Ca^{2+}$ channel antagonist which blocks $Ca^{2+}$ influx. The presynaptic agonist and the $Ca^{2+}$ channel antagonist can attenuate the toxicity. In summary, our model predicts that the toxicity is related to increase in intracelluar $Ca^{2+}$ by 3 separate but related mechanisms: (1) blockade of $Ca^{2+}$ efflux by inhibition of the reuptake transporter; (2) release of stored intracellular pools of $Ca^{2+}$ by a 5-$HT_2$ receptor linked to PI-production; and (3) influx of extracellular $Ca^{2+}$ coupled to release of vesicular 5-HT.

## DISCUSSION

The microculture system we have developed for primary dissociated serotonergic neurons permitted detailed analysis of the MDMA action. The application of the enantiomers of MDMA showed that the S(+) enantiomer was approximately 10-fold more potent at inhibiting the development of [$^3$H]5-HT uptake capacity normally seen in our microcultures. The S(+)-MDMA has more potency in in vitro release studies[34] in producing long-term reductions in 5-HT[45] and in its psychotrophic action[34]. Thus, the results obtained in the microculture system are consistent with the actions of MDMA observed anatomically and functionally with whole animals studies. Furthermore, the long-term changes in 5-HT levels seen after treatment with MDMA are consistent with the decreases seen in 5-HT immunoreactive fiber density in cortex and hippocampus[35]. In our microculture system MDMA at a concentration of $10^{-5}$ M produced a marked decrease in the number of 5-HT-IR cells and in the fiber length of the surviving cells[54]. Thus, biochemical results obtained both in vivo and in vitro are indicative of neurodegenerative changes occurring in the 5-HT neurons. The observation of actual neuron loss in the microculture system is consistent with the greater vulnerability of cultured fetal neurons compared to adult brain neurons in response to both MPTP[31] and methamphetamine[26].

MDMA binds with moderately high affinity (0.61 $\mu$M $K_i$ against [$^3$H]paroxetine) to the 5-HT uptake site[4]. MDMA blocks the in vitro reuptake of [$^3$H]5-HT[47], the S(+)-enantiomer is 3–10 times more potent than the R(−)-enantiomer[37,50]. The ability of MDMA to block the reuptake of 5-HT is a common property of most of the amphetamine compounds[17,18] and is taken as evidence that the release of transmitter produced by these compounds is through the membrane transporter which is $Ca^{2+}$-independent. In fact, 5-HT reuptake blockers which have little effect on 5-HT release (fluoxetine and citalopram) are able to inhibit the MDMA-induced release of 5-HT[4,24,44]. In vivo, citalopram if injected simultaneously with MDMA, can block the long-term degenerative changes which are otherwise observed in the 5-HT system[5]. Fluoxetine was able to protect the cultured serotonergic neurons when added with S(+)MDMA at time zero. This protective effect of reuptake blockers indicates that prevention of the membrane transporter release inhibits the development of the neurotoxicity both in vivo and in culture.

MDMA has been demonstrated to bind to a 5-$HT_2$ receptor with a lower affinity than for its binding to the 5-HT uptake site[4,19]. In culture, we have been able to attenuate the toxicity by co-administration of ketanserin, a 5-$HT_2$ receptor antagonist (Table I). Activation of the 5-$HT_2$ receptor is known to stimulate a PI-hydrolysis second messenger system which should result in release of intracellular stores of calcium[12]. Application of caffeine, which promotes a release of intracellular $Ca^{2+}$ (ref. 52), should potentiate the neurotoxicity if a $Ca^{2+}$-dependent mechanism is involved. In fact, caffeine at $10^{-6}$ M produced a significant potentiation of the inhibition seen by S(+) MDMA at a concentration of $10^{-6}$ M (Fig. 2).

The suggestion that intracellular homeostatic storage of $Ca^{2+}$ may be compromised by MDMA raises the possibility that $Ca^{2+}$-dependent vesicular release of 5-HT may be activated as a secondary response to the drug. Vesicular release of transmitter is produced by depolarization and result in a corresponding influx of extracellular $Ca^{2+}$ (refs. 13, 21, 29). This possible contribution to the MDMA-related toxicity was approached by testing

if presynaptic receptor agonists were effective in attenuating the toxicity. The $\alpha_2$ and $D_1$ agonists are known to be effective at reducing the depolarization induced $Ca^{2+}$-dependent release of 5-HT[6,14,25,38], but they are not operational when 5-HT is released via a $Ca^{2+}$-independent mechanism[27]. Our results on the effects of MDMA on cultured 5-HT neurons show that both B-HT 920 (a specific $\alpha_2$ agonist[36]) and SKF-38393 (a specific $D_1$ agonist[6]) were effective at attenuating the toxicity of S(+)-MDMA at $10^{-5}$ M and completely reverse the inhibition at $10^{-6}$ M. These results strongly suggest that $Ca^{2+}$-dependent release is partially responsible for MDMA neurotoxicity.

The final experiment we performed used a 1,4-dihydropyridine, nimodipine, to close the slow inactivating $Ca^{2+}$ channel[11]. This channel appears to be responsible for the $Ca^{2+}$ influx related to high potassium depolarization[15], and does not interfere with the functioning of the membrane transporter system[8,23]. Nimodipine, at $10^{-9}$ M, was effective at blocking both the R(−) and S(+)-MDMA decrease in cultured serotonergic maturation. This result is consistent with our findings with the presynaptic agonists and implicates a $Ca^{2+}$ influx in the MDMA neurotoxicity.

A calcium-dependent mechanism of irreversible cell death has been described for a variety of cell types[49]. In neurons, excitatory amino acids can produce a sufficient influx of extracellular $Ca^{2+}$ to kill the cell[9,30]. Recent evidence indicates that PCP and MK-801 can attenuate the toxicity of methamphetamine on dopamine cells by their action on the NMDA receptor linked to $Ca^{2+}$ influx[48]. Furthermore, in invertebrate neurons, depolarization and application of 5-HT results in an increase in intracellular $Ca^{2+}$ which produces neurite retraction[10]. These studies indicate that the $Ca^{2+}$ homeostasis inside the cell can be altered to produce neurodegenerative events. We can propose a model of MDMA-induced $Ca^{2+}$-dependent toxicity, which incorporates both cytoplasmic and vesicular release of 5-HT as well as release of $Ca^{2+}$ from intracellular pools (see Fig. 5). Our work indicates that intracellular measures of free $Ca^{2+}$ levels are required to more fully understand the mechanisms of neurotoxicity of a wide range of drugs of abuse.

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