11. A TISSUE CULTURE MODEL OF MDMA TOXICITY

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1. INTRODUCTION

Tissue culture of central nervous system neurons gives a unique opportunity to study both toxic and trophic effects of drugs and other substances on the growth and development of these cells. The substances under examination can simply be added to the culture media, and detailed dose-response and time-course relationships can be studied in a way that no other system affords. Moreover, by altering the cellular composition of the cultures, it can be determined if the drug is acting on a presynaptic or postsynaptic site or even if the effect is mediated through a non-neuronal cell, such as astrocytes [1].

Admittedly, there are shortcomings with the method. For example, direct application of the drugs under investigation to the cultures might result in concentrations that would never be attained in vivo. However, for studying the toxic effects of the isomers of MDMA, tissue culture is a useful system because it can be used to explore the mechanism, on a cellular level, of the toxicity that has already been observed in vivo [2].

We have approached the problem of MDMA toxicity to serotonergic neurons by first observing the effects of various concentrations of each isomer on the growth of these neurons in primary cultures derived from fetal rat brains. From there we have determined the cellular mediators of the toxicity by removing astrocytes (using an anti-mitotic agent, floxuridine, FUDR) or by adding a target tissue to the cultures. In this case, we have chosen to use hippocampus.

Peroutka S.J. (ed), Ecstasy.

After determining the concentration of MDMA that is toxic to the cultures, we have used information from animal studies to predict which subcellular constituents of the cultures would be affected at that concentration and would thus produce the toxicity. For example, MDMA can affect the serotonergic system through actions on the release [3] or re-uptake [4] of the neurotransmitter. There have also been several monoamine receptor interactions reported [5]. To test the individual components of MDMA action, therefore, we devised experiments that were either pharmacologically mimicking or pharmacologically antagonistic. To test the effects of releasing agents, we used p-chloroamphetamine (PCA), and for uptake inhibition, we used fluoxetine. Finally, to test for specific receptor interactions, we observed the effects of MDMA in the presence of mianserin (a 5-HT_2 antagonist), chlorpheniramine or terfenadine (histamine antagonists), or BHT 920 (an alpha2 agonist).

2. TISSUE CULTURE SYSTEM

2.1. Culture of neurons

Embryos at 15 days of gestation were removed by Caesarean section from time-mated pregnant rats (Hilltop Breeding Laboratories). The brains were removed and placed in Eagle's minimum essential media with 1% glucose. A raphe slice that contains the majority of the midbrain serotonergic cell bodies was dissected [6].

Briefly, two scalpels were used to transect the fetal brain at the pontine and mesencephalic flexures. A cut was made through the cerebral aqueduct to expose the tegmentum. Two longitudinal cuts were made approximately 0.5 mm off the midline. The underlying meninges were removed and the strip transferred to Ca^{++}/Mg^{++} free MEM containing 1% glucose.

The strips were transferred to 5–10 ml of Versene (Gibco brand EDTA) solution and gently agitated by repeated trituration using a fire-polished Pasteur pipette. The cell suspension was spun at 500 g for five minutes and the pellet resuspended in complete media (MEM with non-essential amino acids, 1% glucose, and 5% fetal calf serum) before being spun again. The final pellet was resuspended in complete media (approximately 0.5 cc/raphe strip). The complete media consisted of MEM with non-essential amino acids, 1% glucose, and 10% fetal calf serum.

The cells were plated onto 96-well Linbro plates (Falcon Labware) previously coated with poly-l-lysine (25 mcg/ml) for quantitation by specific uptake of serotonin, or onto 8-well slides (Titer Tek) previously coated with poly-l-lysine for morphological assessment using immunocytochemistry (see below).

The cells were plated at initial plating densities averaging 1.2×10^6 cells/cm^2. In most experiments, the cells were maintained in an incubator at 37° in an atmosphere of 95%/5% CO_2/O_2 for five days. Drugs to be tested were added aseptically either at the time of plating or at various timepoints throughout the five days. Three to four wells were used for each drug condi-

tion in at least two separate experiments. In any one experiment, up to twenty-three concentrations of a drug could be tested, generally in a range from 10^{-15} to 10^{-3} M.

2.2. Cellular composition

As a model of a complete serotonergic synapse, cultures of raphe neurons were co-cultured with cells from the hippocampus. Briefly, the hippocampal regions from 18 day rat fetuses were dissected out and freed of meninges and choroid plexus. The tissue was minced and dissociated as described for raphe cells. Using this method, we have shown that development of the serotonergic neurons is enhanced by presence of target regions [1].

Since the toxicity of the dopaminergic toxin MPTP is mediated via astroglial cells, we tested the role of astroglial cells in MDMA toxicity by incubating cultures with an anti-mitotic, floxuridine (FUDR). Astroglial cells are actively dividing in our cultures and are therefore inhibited or destroyed by the addition of 20 microgm/ml FUDR to the culture media. We have found that this addition has little if any effect on the serotonergic neurons during the short period of time we have been growing the cultures.

2.3. Quantitation of growth/toxicity

Immunocytochemistry was performed using a specific antibody raised against serotonin conjugated to hemocyanin. The cultures were pretreated with 10^{-4} pargyline for 30 minutes, followed by 10^{-5} L-tryptophan for one hour. Cultures were washed with tris-buffered saline (TBS) and fixed with 4% paraformaldehyde containing 0.05% $MgCl_2$ and 5% glucose. After two hours fixation, the cells were rinsed with TBS and processed using the ABC kit (Vectastain) with DAB as substrate. Morphological analysis was performed on ten randomly selected cells from coded cultures for each culture condition. The somal area and cumulative process length were calculated using a Bioquant Computer System IV (R&M Biometrics, Inc.) with a Leitz Diaplan microscope with a 25X Pl-apo objective.

Cultures (in 96-well plates) were washed with MEM, and fresh MEM with 0.5% glucose and 50 nM ^{3}H-5-HT (NEN, 26 Ci/mmole) was added for twenty minutes at 37%. Non-specific uptake was determined in the presence of 10^{-5} M fluoxetine. After incubation, cultures were washed twice with MEM and retained radiolabel extracted with absolute ethanol and counted in a Beckman liquid scintillation counter. Results (expressed as cpm/well) were statistically analyzed and plotted using Sigma Plot (Version 3; Jandel Scientific).

3. MDMA EFFECTS ON CULTURED NEURONS

Figure 1 shows the specific uptake of serotonin in cultures of neurons exposed to the isomers of MDMA. The approximate K_i for toxicity is 8 micromolar for *S*-(+)-MDMA and 90 micromolar for *R*-(−)-MDMA. Morphometric

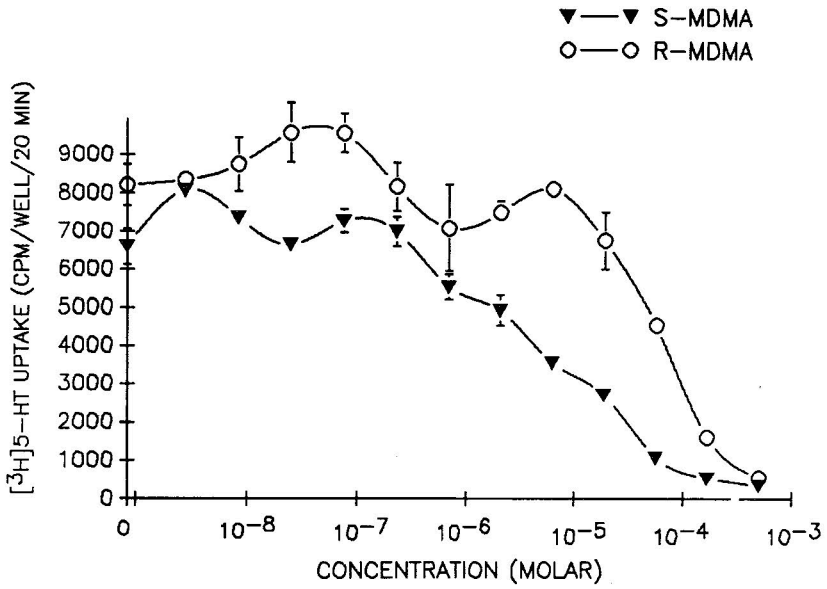


Figure 1. Effects of various concentrations of the isomers of MDMA on specific uptake of [^{3}H]-serotonin into primary cultures of serotonergic neurons maintained in culture for four days. Each point represents the mean and SEM from four cultures.

analysis (Table 1) showed significant degeneration of serotonergic cell bodies at an MDMA concentration of 5×10^{-4} but not 5×10^{-7} M. Moreover, exposure of cultures to MDMA not only reduced cell number but also decreased the process outgrowth from those neurons which remained (Figure 2).

In studies pharmacologically mimicking the known actions of MDMA, the releasing agent p-chloroamphetamine (PCA) was examined (Figure 3) and found to have an approximate K_i of 1×10^{-5} M. The specific serotonin uptake inhibitor fluoxetine, was also found to be toxic (Figure 3), with an approximate K_i of 100 nM. However, at lower (non-toxic) doses (10^{-7} M), fluoxetine attenuated the toxicity of 10^{-4} MDMA (Table 2).

Table 1. Effects of the stereoisomers of MDMA on survival of serotonergic neurons in tissue culture.

	Number of cells/unit area
Control	8.7 ± 1.5
S(+)-MDMA: 10^{-10} M	8.7 ± 1.5
5×10^{-7} M	9.8 ± .15
5×10^{-4} M	2.3 ± .8
R(−)-MDMA: 10^{-10} M	8.5 ± .2
5×10^{-7} M	9.5 ± 2
5×10^{-4} M	3.5 ± .5

In studies pharmacologically blocking the known effects of MDMA, we examined antagonists for the following receptors: serotonin$_2$ (mianserin), histamine (chlorpheniramine and terfenadine), and the alpha$_2$ agonist BHT 920. Since there has also been a report on MDMA causing release of dopamine, we studied the effects of the dopamine uptake inhibitor nomifensine. Toxicity was not attenuated by any of these agents, with the exception of BHT 920. At a concentration of 10^{-8} M, BHT 929 inhibited the toxicity of *S*-(+)-MDMA by 49% and *R*-(−)-MDMA by 34%. All of these results are given in Table 3.

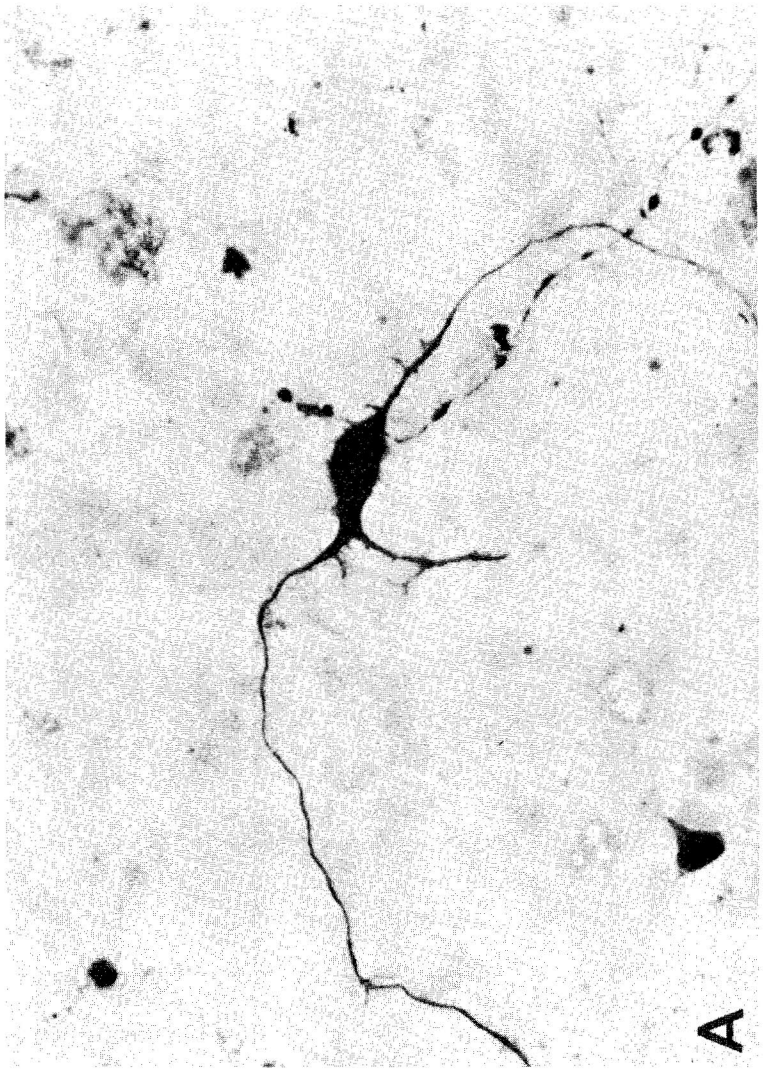
The final series of experiments aimed at localizing the cellular site of toxicity showed that toxicity is attenuated by either postsynaptic tissues or astroglial cells. In co-cultures of raphe cells with hippocampus, the toxicity curves are shifted such that the two isomers have identical profiles; that is, the *S*(+) isomer becomes 10X less toxic (Figure 4). In cultures that have been treated with FUDR, MDMA toxicity is greatly increased (Figure 5).

4. CONCLUSIONS

Using a tissue culture model of serotonergic neurons, we have studied the mechanism of MDMA toxicity. Although our work is still in progress, we can draw some preliminary conclusions. First, the *S*(+) isomer of MDMA is approximately ten times more potent in producing toxicity than is the *R*(−) isomer. This is comparable to results in vivo, which have also indicated that this is the more toxic isomer [3]. However, the in vivo studies indicate that the *R*(−) isomer is only toxic (ie., causes reduction of serotonin) acutely and that one week after treatment, control levels of serotonin are found. Conversely, the *S*(+) isomer produces a long-lasting depletion. This discrepancy could be related to the fact that our model allows the neurons to be left in contact with the MDMA for four days without a washout period, before measuring serotonin uptake. Also, it is possible that the recovery of serotonin levels would occur in our cultures, as well, if they were maintained for one week. Both of these issues will be addressed in future experiments, as they will give important information on the two forms of toxicity that appear to occur.

One notable difference between the isomers of MDMA, is that *S*(+)-MDMA is approximately tenfold more potent on the release of serotonin. This then seems a likely candidate to at least partially explain the toxicity of MDMA. There is further evidence from other drugs we have tested.

The only receptor-active drug that we have tried and that did have an effect is the alpha$_2$ agonist BHT 920. This drug inhibited the toxicity produced by either isomer with an approximate K_i value of 10 nM. Alpha$_2$ adrenergic receptors are known to occur on serotonergic neurons and to be inhibitory to release to serotonin [7]. Thus inhibition of release of serotonin appears to be one mechanism by which toxicity is attenuated. This would be consistent with the observation that the serotonin releaser PCA is also toxic. Whether or not release is mediated by MDMA through an action on the same receptor (for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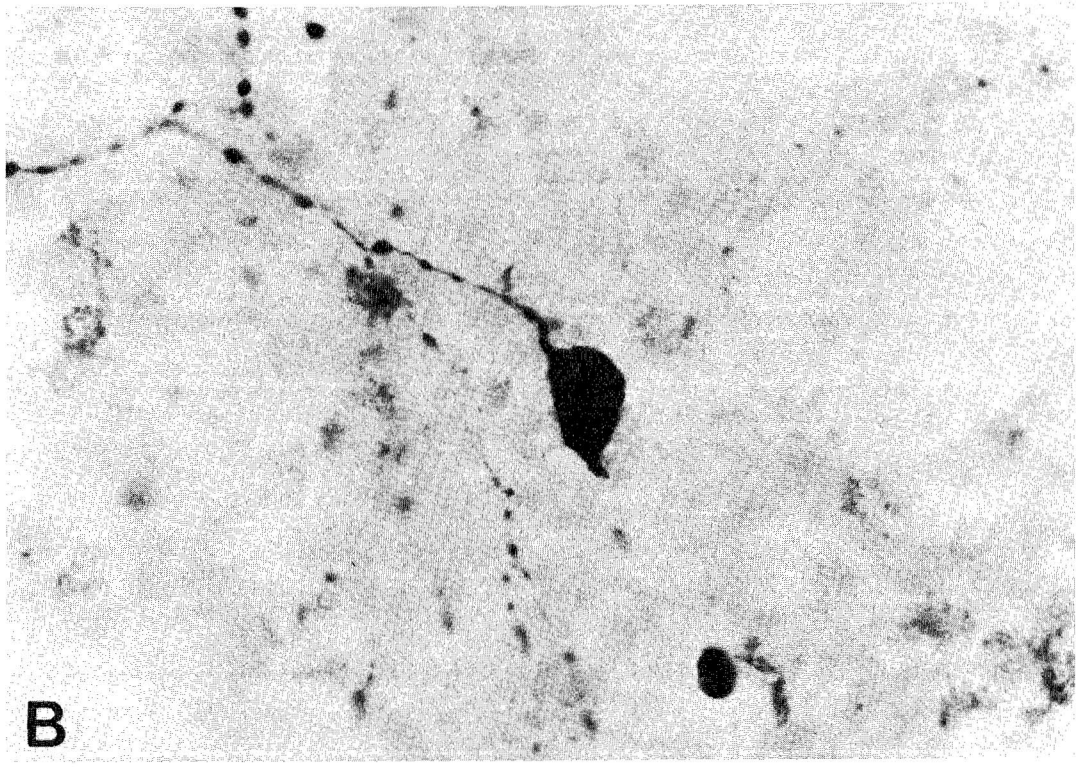

Figure 2. Immunocytochemically stained serotonergic neurons grown in culture for four days without (A) or with (B) 10^{-4} M MDMA.

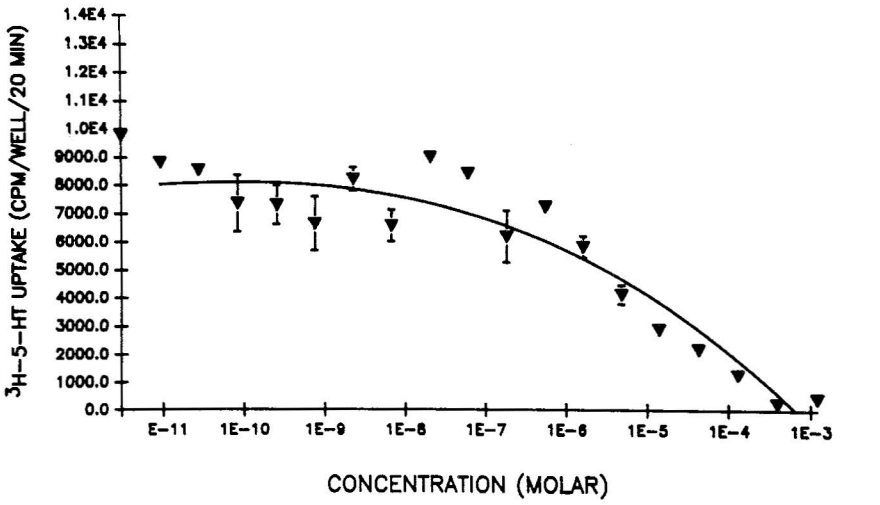

Figure 3. Effects of various concentrations of p-chloramphetamine (PCA) on specific uptake of [^{3}H]-serotonin into primary cultures of serotonin neurons maintained in culture for four days. Each point represents the mean and SEM from four cultures.

Table 2. Effect of the serotonin uptake inhibitor fluoxetine on MDMA toxicity to serotonin neurons.

	Uptake of [^{3}H]-serotonin, cpm		
		Fluoxetine	
	Control	10^{-7}M	10^{-5}M
Control	773 ± 93	723 ±86	289 ± 57
S-MDMA (10^{-4}M)	184 ± 35	969 ± 32	384 ± 148
R-MDMA (10^{-4}M)	267 ± 68	661 ± 84	–

Table 3. Effects of various pharmacological agents on 10^{-4}M MDMA-induced toxicity.

	Specific uptake of [^{3}H]-serotonin cpm/well			
EXPERIMENT A:	Control	Mianserin 10^{-8}	Chlorpheniramine 10^{-6}	Terfenadine 10^{-6}
Control	5299 ± 595	5743 ± 958	5804 ± 539	5888 ± 477
S(+)MDMA	611 ± 79	731 ± 65	898 ± 54	702 ± 79
R(−)MDMA	1180 ± 54	998 ± 77	762 ± 70	1048 ± 148
EXPERIMENT B:	Control	Nomifensine 10^{-6}	BHT-920 10^{-8}	
Control	3028 ± 250	3285 ± 50	3659 ±560	
S(+)MDMA	458 ± 59	610 ± 163	1707 ± 259	
R(−)MDMA	1403 ± 86	928 ±202	1788 ± 98	

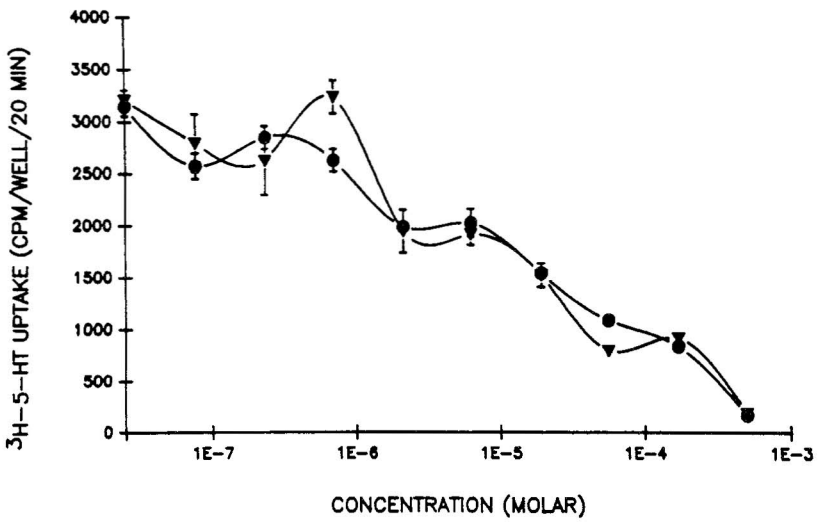

Figure 4. Effects of the *S*(+)-isomer (triangles) and the *R*(−)-isomer (circles) of MDMA on the specific uptake of [^{3}H]-serotonin into serotonergic neurons grown in tissue culture in the presence of hippocampus for four days. Each point represents the mean and SEM of four cultures.

which it has an apparent K_i of 3.6 micromolar) can only be speculated at this time.

Our results with fluoxetine also suggest a role for release in the toxicity. Concentrations of fluoxetine that protect the neurons from MDMA presumably inhibit its transport into the neurons. Thus internalization of the drug appears to be important. It has also been shown in vivo that serotonin uptake inhibitors, such as citalopram [3] and fluoxetine [8], inhibit the toxic actions of MDMA. The higher concentrations of fluoxetine are presemably toxic due to an effect of this drug on systems other than transport.

Our morphological studies indicate the actual loss of cells, although this finding is not clearly shown in vivo. The cell death in our cultures could be due to the fact that these are immature cells that are highly dependent on terminal integrity for survival. However, it could also be indicative of a process that occurs with repeated exposure to the drug.

Our work with neurotransmitter receptor blockers is not yet complete; however, we have eliminated the possible contribution to toxicity made by a number of receptors. Specifically, the lack of protection afforded by mianserin has ruled out an action on the serotonin$_2$ receptor. Similarly, the lack of attenuation by the histamine antagonists terfenadine and chlorpheniramine seems to rule out these receptors.

Our final experiments on the cellular localization of toxicity indicate that the effects of MDMA are directly on the serotonergic nerve terminal and that an intermediate toxin is not produced by other cells. This mechanism of toxicity

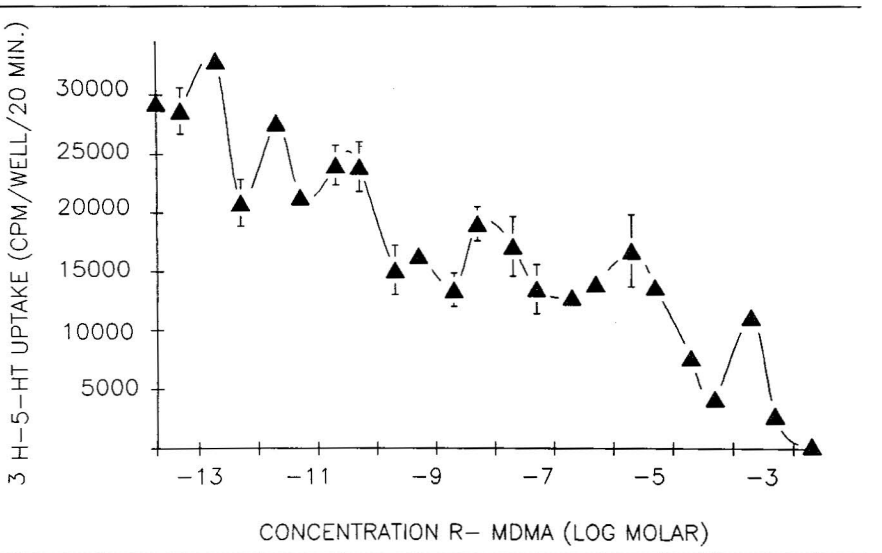

Figure 5. Effects of various concentrations of *R*(−)-MDMA on specific uptake of [^{3}H]-serotonin into primary cultures of serotonin neurons grown in culture for four days after the addition of the antimitotic agent FUDR. Each point represents the mean and SEM of four cultures.

had been shown for MPTP, which is metabolized by astroglial cells into the dopamine toxin MPP^+. Interestingly, the work with FUDR-treated cultures indicates that astroglial cells are, in fact, protective of the serotonin terminal. This may be due to uptake of MDMA into astrocytes, which contain specific high affinity carrier systems for a number of neurotransmitters, including serotonin [9].

The role of target tissue in attenuating the toxicity of the *S*(+)-isomer is not readily explained. In previous studies [1], we have shown that the presence of target tissue is stimulatory to growth of the neurons either by providing trophic factors or by inhibiting toxic factors. The results with MDMA suggest that inhibition of toxic substances is indeed possible. Regardless of the mechanism of toxicity attenuation by target, our findings further point out the possibility that two mechanisms of toxicity are in force and that the *S*(+)-isomer has more long-lasting effects by virtue of eliciting both, whereas the *R*(−)-isomer only elicits one [3].

In conclusion, we have applied the methodology of tissue culture to the problem of MDMA-induced serotonergic neuronal degeneration. We have been able to replicate several in vivo observations in our model, such as the differences between the isomers and the protective effects of fluoxetine. In addition, we have made some new observations on the role of target tissue and astrocytes in attenuating the toxicity, and raised the possibility that chronic exposure may actually lead to cell death. Finally, we have shown a role for

release-regulating receptors and that actions on serotonin$_2$ or histamine receptors are not involved.

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