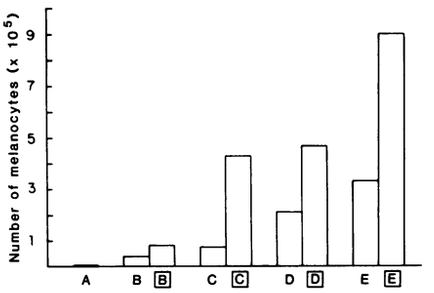

Fig. 2. Influence of added TPA on the melanocyte growth-promoting activity of cell extracts. Melanocytes (see below) were cultured for 20 days in cMEM with the following supplements: A, control (no supplement); B, TPA, and B, TPA plus cholera toxin; C, SK-MEL-131 extract, and C, SK-MEL-131 extract plus TPA; D, AO_2V_4 astrocytoma extract, and D, AO_2V_4 astrocytoma extract plus TPA; E, WI-38 fibroblast extract, and E, WI-38 fibroblast extract plus TPA. Amounts were TPA, 10 ng/ml; cholera toxin, $10^{-8}M$; and cell extracts diluted 1:1000. An epidermal cell suspension derived from a human foreskin (1290) was seeded into tissue culture cluster dishes (12 wells with 2×10^5 cells per well) in cMEM and the factors listed above. Twenty-four hours later, all unattached cells were removed, and each well was refed with the appropriate medium. Three days later the cells were trypsinized and seeded at approximately 1×10^4 cells per well into tissue culture cluster dishes (six wells). Melanocytes were allowed to attach, and later geneticin (100 μg/ml) was added for an overnight treatment (*3*). After treatment, cells were grown for 20 days, trypsinized, and counted. Contamination by fibroblasts was excluded by staining for leucine aminopeptidase–positive cells.

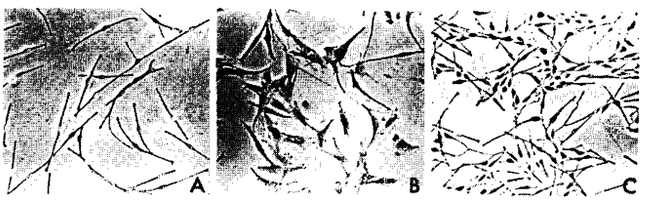

Fig. 3. Morphology of melanocytes cultured in medium containing TPA or melanocyte growth factors derived from the astrocytoma cell line AO_2V_4. (A) TPA, 10 ng/ml; (B) AO_2V_4 astrocytoma extract diluted 1:1000; (C) TPA at 10 ng/ml plus AO_2V_4 astrocytoma extract diluted 1:1000 (magnification, ×200).

bined with cholera toxin was added. Factors in the extracts appear therefore to support the growth of individual melanocytes very efficiently and permit their cloning; this cannot be achieved with cultures grown in TPA or TPA and cholera toxin.

Since known growth factors lacked melanocyte growth-promoting activity, we assume that we are dealing with one or a family of new growth factors. Cell extracts appear to be a far richer source of these new factors than culture supernatants, and it will be important to search for growth factors in extracts of other differentiated cell types. Although it is possible that combinations of known growth factors would reproduce the effects observed with extracts, experiments to test this have yielded negative results. As melanomas and astrocytomas originate from neuroectodermal progenitor cells, production of melanocyte growth factors could be a property of selected cell types derived from this lineage. Support for this idea comes from the recent finding that bovine brain extracts contain a factor capable of stimulating melanocyte growth (*7*). With regard to the potent mitogenic effect of WI-38, fibroblasts are known to have both mesodermal and neuroectodermal origin. In addition, the fact that growth-promoting activity could be extracted from skin fibroblasts raises the possibility that dermal fibroblasts participate in the regulation of melanocytes, an idea that has been widely held. A broader range of normal and malignant cells are being tested to determine the relation of cell type to factor production. Defining the nature and number of these melanocyte growth factors represents the next step in the analysis. The factor from WI-38 appears to be acid- and alkaline-stable, heat-labile, and to have a molecular weight of 40,000 by gel filtration; these characteristics distinguish it from known growth factors.

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Hallucinogenic Amphetamine Selectively Destroys Brain Serotonin Nerve Terminals

Abstract. *(±)-3,4-Methylenedioxyamphetamine (MDA), an amphetamine analog with hallucinogenic activity, produced selective long-lasting reductions in the level of serotonin, the number of serotonin uptake sites, and the concentration of 5-hydroxyindoleacetic acid in rat brain. Morphological studies suggested that these neurochemical deficits were due to serotonin nerve terminal degeneration. These results show that MDA has toxic activity for serotonin neurons in rats and raise the question of whether exposure to MDA and related hallucinogenic amphetamines can produce serotonin neurotoxicity in the human brain.*

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(±)-3,4-Methylenedioxyamphetamine (MDA) is a synthetic amphetamine derivative that produces a mixture of psychomotor stimulatory and hallucinogenic effects (*1*). This combination of psychotropic actions may stem from MDA's

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Table 1. Regional brain levels of dopamine, serotonin, and norepinephrine in rats 2 weeks after various doses of (±)-3,4-methylenedioxyamphetamine (MDA). Each dose was administered approximately every 12 hours for four consecutive days. Values (in micrograms per gram of tissue) represent the mean ± standard error of the mean for $n = 4$ except where noted. N.M., not measured because these doses of MDA produced little or no effect on serotonin levels.

MDA dose (milligrams per kilogram)	Striatum		Hippocampus		Rest of brain		
	Dopamine	Serotonin	Norepinephrine	Serotonin	Dopamine	Norepinephrine	Serotonin
1.25†	10.6 ± 0.4	0.42 ± 0.02	N.M.	0.39 ± 0.03	0.17 ± 0.01	N.M.	0.29 ± 0.01
2.5	11.7 ± 0.4	0.37 ± 0.04	N.M.	0.40 ± 0.02	0.18 ± 0.01	N.M.	0.34 ± 0.03
5	12.4 ± 0.7	0.36 ± 0.04	N.M.	0.28 ± 0.04*‡	0.18 ± 0.01	N.M.	0.23 ± 0.03*
10	11.5 ± 0.6	0.18 ± 0.05*	0.31 ± 0.06	0.16 ± 0.05*	0.18 ± 0.02	0.46 ± 0.04	0.19 ± 0.02*
20	10.6 ± 0.4	0.14 ± 0.01*	0.38 ± 0.01	0.10 ± 0.01*	0.17 ± 0.02	0.47 ± 0.01	0.16 ± 0.02*
40	10.8 ± 0.5	0.18 ± 0.01*	0.38 ± 0.04	0.09 ± 0.02*	0.18 ± 0.02	0.50 ± 0.03	0.16 ± 0.02*
Control (saline)	11.6 ± 0.3	0.43 ± 0.05	0.34 ± 0.02	0.41 ± 0.02‡	0.19 ± 0.01	0.46 ± 0.01	0.33 ± 0.04

* $P < 0.05$, determined by individual comparison to control after a simple one-way analysis of variance (ANOVA) showed an F value with $P < 0.05$.
† $n = 3$. ‡ $n = 5$.

close structural relation to both amphetamine, a prototypic stimulant, and mescaline, a well-known hallucinogen. Clinically, MDA has been evaluated as an anorectic and antidepressant and as an adjunct to psychotherapy (*2*). Although some investigators have advocated that MDA be used to facilitate psychotherapy, it has yet to find an accepted place in the medical pharmacopoeia. In contrast, MDA has been a popular illicit drug for more than 20 years (*3*). Despite recognition of MDA's high abuse liability, relatively little research has been done to assess its toxicity. The few studies performed with animals indicate that the toxicity of MDA generally parallels that of amphetamine (*4*). As such, MDA can produce mydriasis, profuse salivation, tachycardia, hypertension, hyperthermia, convulsions, and death. The few studies done with humans suggest that in doses up to 300 mg MDA is free of significant toxicity (*2*). Higher doses have been associated with nearly fatal as well as fatal reactions (*5*). Marked physical exhaustion lasting up to 48 hours after drug ingestion (100 to 300 mg) has also been reported (*6*).

Amphetamines such as (+)-methamphetamine and (+)-amphetamine are toxic to brain dopamine and serotonin neurons (*7*). This toxicity is manifested by long-lasting reduction in the levels of dopamine and serotonin and a decreased number of uptake sites in the brain (*7*). In the case of dopamine neurons, these deficits have been shown to be the result of dopamine nerve terminal degeneration (*8*). In view of these findings and of the paucity of information about MDA toxicity, we evaluated the toxic potential of MDA for dopamine, serotonin, and norepinephrine neurons. We now present chemical and anatomical evidence of selective serotonin nerve terminal degeneration after single or multiple doses of MDA.

We examined the neurotoxic potential of various doses (1.25, 2.5, 5, 10, 20, and 40 mg/kg) of MDA by administering each of these doses subcutaneously to a group of rats every 12 hours for four consecutive days and then measuring brain dopamine, serotonin, and norepinephrine levels 2 weeks after treatment (*9*). Doses were selected to cover a range known to produce from minimum to maximum behavioral effects in rodents (*4*, *10*). Measurements made 2 weeks after drug treatment revealed that MDA decreased serotonin levels in various brain regions without affecting either dopamine or norepinephrine levels in the same regions (Table 1). The lowest dose of MDA that produced this effect was 5 mg/kg. This dose lowered serotonin levels in the hippocampus and in the rest of the brain but not in the striatum (Table 1). Higher doses reduced serotonin levels in all the brain regions examined. However, even the highest dose (40 mg/kg) produced no lethality, and 2 weeks after drug administration MDA-treated rats could not be distinguished from control rats by observation.

We examined two other serotonin neuronal markers after MDA administration. Rats were given 10 mg of MDA per kilogram for 4 days, and 2 weeks later they were killed to measure uptake of ^{3}H-labeled serotonin and the level of 5-hydroxyindoleacetic acid in the hippocampus. Kinetic analysis of ^{3}H-labeled serotonin uptake (*11*) by crude synaptosomal suspensions prepared from the hippocampus of saline- and MDA-treated rats indicated that MDA produced a long-lasting reduction in the maximum velocity (V_{max}) of ^{3}H-labeled serotonin

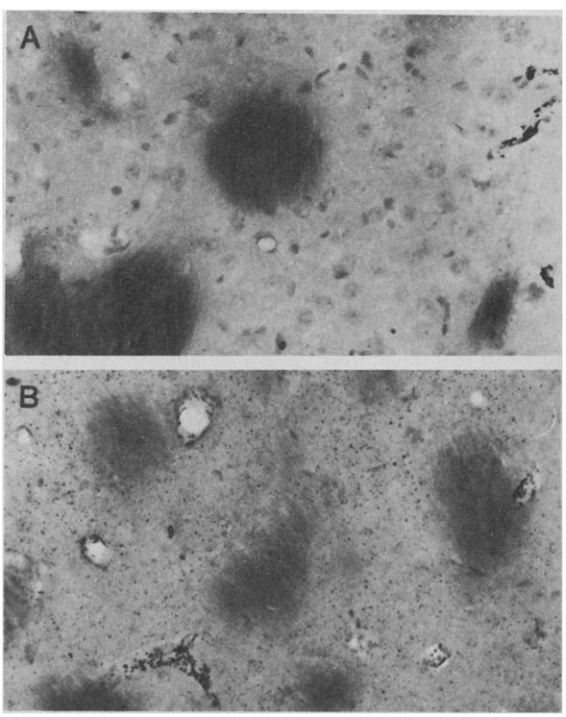

Fig. 1. Silver-stained coronal sections through the striatum of (A) a control rat and (B) a rat given two injections of MDA (10 mg/kg) subcutaneously 12 hours apart. The Fink-Heimer method with cresyl-violet counterstain was used (18-hour survival).

without affecting its affinity constant (K_m) [V_{max}, 7479 ± 678 count/min (control) and 3265 ± 408 count/min (MDA), difference significant at $P < 0.01$; K_m, 0.12 ± 0.03 μM (control) and 0.16 ± 0.04 μM (MDA), not significant]. This result indicates that MDA reduces the number but not the affinity of synaptosomal serotonin uptake sites. MDA also produced a long-lasting reduction in 5-hydroxyindoleacetic acid concentration (*12*) in the hippocampus [0.33 ± 0.03 μg/g (control) and 0.12 ± 0.01 μg/g (MDA), significant at $P < 0.01$]. This finding, along with the observations of decreased serotonin level and uptake after MDA administration, suggests that MDA is toxic to serotonin neurons.

To confirm this, we looked for evidence of serotonin nerve terminal destruction after MDA administration using the Fink-Heimer method (*13*), which allows for selective silver impregnation of degenerating axons and terminals. With this method, degenerating nerve terminals were found in the hippocampus and striatum of all three rats given MDA (Fig. 1). No such terminal degeneration was found in any of the three control rats. Because the hippocampus and striatum are the brain regions in which MDA produced selective long-lasting serotonin depletions (Table 1), it seems reasonable that the degenerating nerve terminals in Fig. 1 are serotonergic and that MDA induces prolonged serotonin neurochemical deficits by destroying serotonin nerve terminals.

In a final experiment, groups of rats ($n = 4$) were given 10 mg of MDA per kilogram every 12 hours for 4, 2, 1, and 0.5 days and then killed 2 weeks later. Determinations of serotonin levels at this time revealed that a single injection of MDA (0.5-day regimen) reduced hippocampal serotonin content by 32 percent and that additional injections led to greater deficits (Table 2).

Our study raises the question of whether MDA is toxic to serotonin neurons in humans. Because of the differences in species, dose, frequency, and route of administration, as well as differences in the way in which rats and humans metabolize amphetamine (*14*), it would be premature to extrapolate our findings to humans. Also, the doses of MDA required for neurotoxicity in the rat (5 to 10 mg/kg, Tables 1 and 2) are roughly three to five times higher than those required to produce hallucinogenic effects in humans (approximately 1.5 to 3 mg/kg) (*1, 2*). Hence, the doses of MDA typically ingested by humans may not be sufficiently high to induce serotonin neurotoxicity, unless humans are more sensitive than rats to the toxic effects of MDA. That this may be the case is suggested by the observation that an MDA dose of 7.5 mg/kg approaches the lethal dose in humans (*5*), whereas in rats even a dose of 40 mg/kg did not produce any lethality.

Table 2. Serotonin content of rat hippocampus 2 weeks after various regimens of MDA administration (10 mg/kg). Values (in micrograms per gram of tissue) represent the mean ± standard error of the mean ($n = 4$). All values were significantly different ($P < 0.05$, one-way analysis of variance) from control.

Duration of regimen (days)	Serotonin content	Decrease (%)
0.5	0.28 ± 0.04	32
1	0.17 ± 0.01	59
2	0.12 ± 0.01	74
4	0.10 ± 0.01	76
Control	0.41 ± 0.02	

Other ring-substituted amphetamines such as 3,4-methylenedioxymethamphetamine, 3,4,5-trimethoxyamphetamine, and 2,5-dimethoxy-4-methylamphetamine are widely abused, and possible toxic effects on serotonin neurons of these ring-substituted amphetamines need to be evaluated. Such studies should help identify the structural requirements for a ring-substituted amphetamine to produce serotonin neurotoxicity. A better understanding of such structure-activity relations could be of value in suggesting ways in which endogenous substances (such as biogenic amines and free phenylethylamines) structurally related to MDA and other toxic amphetamines may be modified in vivo into neurotoxic compounds. Such endogenously formed neurotoxins (*15*) could play a role in the etiology of neurodegenerative disorders involving monoamine-containing neurons in the central nervous system of humans.

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9. Male albino Sprague-Dawley rats weighing approximately 250 g (Holtzman) were housed singly in suspended wire-mesh cages with free access to food (Purina Rat Chow) and water in a colony room maintained at 23° ± 1°C. Purity of (±)-MDA hydrochloride (National Institute on Drug Abuse) was confirmed by means of mass spectroscopic analysis. MDA was administered subcutaneously after being dissolved in sterile 0.9 percent saline at various desired concentrations. Dose (expressed as the free base) was adjusted by injecting each of these MDA solutions on a 1 ml/kg basis. Control rats were injected with an equal volume of saline. Regional brain dopamine, serotonin, and norepinephrine levels were determined by high-performance liquid chromatography (HPLC) coupled with electrochemical detection. Dopamine and serotonin were measured as described [R. Keller, A. Oke, I. Mefford, R. Adams, *Life Sci.* **19**, 995 (1976)] with modification [J. Lucot, J. Horwitz, L. S. Seiden, *J. Pharmacol. Exp. Ther.* **217**, 738 (1981)]. Norepinephrine was analyzed as described [R. S. Fenn, S. Siggia, D. J. Curran, *Anal. Chem.* **50**, 1067 (1978)].
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12. Levels of 5-hydroxyindoleacetic acid were measured by reversed-phase HPLC as described [C. Kotake, G. Vosmer, T. Heffner, L. Seiden, *Pharmacol. Biochem. Behav.* **22**, 85 (1985)].
13. Before performing terminal degeneration studies in MDA-treated rats, we assessed the Fink-Heimer method for its ability to show serotonin terminal degeneration. 5,7-Dihydroxytryptamine (75 μg) was dissolved in 0.9 percent NaCl containing ascorbic acid and injected into the left lateral ventricle. Eighteen hours later terminal degeneration was found in both the hippocampus and striatum. This short survival time seems critical because terminal degeneration is not observed after longer survival times [V. J. Massari, Y. Tizabi, E. Sanders-Bush, *Neuropharmacology* **17**, 54 (1978)].
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