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Neuropathological Evaluation by Combined Immunohistochemistry and Degeneration-Specific Methods: Application to Methylenedioxymethamphetamine

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ABSTRACT: *To best interpret the significance of neurological alterations produced by chemicals, the changes in morphological as well as neurochemical parameters must be measured and compared to each other. We have devised an approach to readily label microscopic sections for multiple antigens (neurotransmitters, enzymes, peptides, etc.) as well as for the demonstration of degenerating structures by silver impregnation. Here, we applied silver-staining together with immunolabelling of 5-HT and tyrosine hydroxylase (the rate-limiting enzyme for dopamine synthesis) to study neurohistological alterations produced by methylenedioxymethamphetamine (MDMA), a hallucinogenic stimulant previously used as an adjunct to psychotherapy and now a popular recreational drug ("Ecstasy"). Single oral doses of 40 or 80 mg/kg MDMA doubled the density of silver-impregnated (degenerating) fiber terminals in the caudate nucleus compared to controls, when rats were sacrificed 18 hours after treatment. Four months after two 40 mg/kg oral doses per day for four days, rats had a reduced neurochemical content of 5-HT in the hippocampus, and fewer immunostainable 5-HT axons per unit area in the hippocampal stratum lacunosum but no change in brain-stem neurochemical 5-HT content or in the numeric density of 5-HT-positive cell bodies in the dorsal raphe nucleus. The neurohistology suggests interpreting the changes in neurochemical content of serotonin produced by MDMA as due to degeneration followed by subsequent loss of 5-HT axons, rather than a decrease in the rate of neuronal 5-HT synthesis or a toxicity directed toward 5-HT cell bodies. The combination of neurohistological and neurochemical evaluation will continue to prove useful in comprehensive evaluation of the neurological effects of chemical exposure.*

Key Words: MDMA, "Ecstasy", Serotonin, Immunohistochemistry, Neuropathology, Degeneration, Histology

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INTRODUCTION

Methylenedioxymethamphetamine (MDMA or "Ecstasy") is a hallucinogenic stimulant and has been used by some health professionals in the field of psychotherapy and by the general public as a recreational drug (Frith *et al.*, 1987). MDMA has recently been reported to produce neurochemical effects in the rat after subcutaneous (Gibb *et al.*, 1986; Schmidt, 1987) or oral administration (Slikker *et al.*, 1986; 1988). MDMA decreased tryptophan hydroxylase (the rate-limiting enzyme of serotonin synthesis) activity in the hippocampus, frontal cortex, and striatum of the rat (Gibb *et al.*, 1986). MDMA also reduced the content of 5-HT itself as well as its metabolite (5-HIAA) in several brain regions (Schmidt, 1987; Slikker *et al.*, 1986; 1988) in a biphasic manner; an initial early decrement returned to control levels by 24 hr, while a subsequent decrease lasted at least 7 days (Schmidt, 1987) and up to one month in the study of Slikker *et al.*, (1986; 1988). A closely-related structural analog, methylenedioxyamphetamine (MDA), has been reported to cause nerve terminal degeneration in the striatum and hippocampus of treated rats studied by the silver-degeneration staining approach (Ricaurte *et al.*, 1985).

These initial findings raise several important questions. First, do the neurochemical changes produced by MDMA represent damage to neuronal structural elements? Secondly, are the effects of MDMA even more long-lasting than one month? Thirdly, are these effects selective for elements that can be immunohistochemically identified as serotonergic? And finally, are any effects selective for particular neuronal elements of the serotonergic system, i.e., dendrites, soma, or axons? We designed this study to address these questions in the rat by combining a neurohistological procedure that selectively stains only degenerating neuronal elements with immunohistochemical and neurochemical procedures.

METHODS

Animals (adult male Sprague-Dawley rats, Charles River, Portage, Michigan, Expt 1; adult female Sprague-Dawley derived NCTR strain CD rats, Expt 2) were treated with MDMA as previously described (Frith *et al.*, 1987). The treatment protocols employed oral dosing by gavage with either 40 or 80 mg/kg of MDMA, as detailed in Table 1. Animals of the second experiment only were pre-treated one hour before sacrifice with 100 mg/kg of the serotonin dietary precursor l-tryptophan (Fluka Chemical Corp., F.R.G.) to enhance (Cooper *et al.*, 1982) the immunohistochemical staining of 5-HT-containing neuronal elements. Animals studied by neurohistology were anesthetized with 60 mg/kg of sodium pentobarbital and sacrificed by fixation with 10% phosphate-buffered formalin perfused through the ascending aorta. Brains were removed the same day as sacrifice and stored overnight in fixative. To eliminate possible effects of length of storage of brains in fixative, all rats of an experiment were sacrificed on the same day, and their brains were sectioned the following day utilizing a Vibratome (Technical Products, Inc. St. Louis Missouri). Thirty-micron sections were collected in 0.1 M phosphate buffer (pH 7.4) and stored in 24-well plastic tissue culture dishes (Costar, Inc. Cambridge, Massachusetts) in either this same buffer (for immunohistochemistry as described below based on the methods of Sternberger, 1982) or in 10% phosphate-buffered formalin (for the degeneration-specific silver stain described by Nadler and Evenson, 1983) until histological processing within the next two weeks.

In both experiments, a series of coronal sections through the caudate nucleus about at the level of bregma (Paxinos and Watson, 1982) as well as sections through the hippocampus about 3.5 mm posterior to bregma were stained. Two sections per animal for each type of stain and for each brain area were evaluated. Included also in the regions evaluated in Experiment 2 were sections through the dorsal nucleus of the raphe (about 7.8 mm posterior to bregma), which contains the cell bodies of origin of most of the serotonin terminals of cortex, caudate, and hippocampus (Cooper *et al.*, 1982). Balanced blocks containing an equal number of sections from each animal of each experimental group (for each brain area separately) were stained in a 24-compartment basket floored with nylon mesh. This procedure equated all incubations

TABLE 1. Experimental Design for MDMA Neurohistology.

Group	n	Treatment (p.o.)	Time of Sacrifice *
Experiment 1			
1	3	8 x 80 mg/kg MDMA**	2 weeks
2	3	1 x 80 mg/kg MDMA	18 hours
3	3	1 x 40 mg/kg MDMA	18 hours
4	2	1 x 10 ml/kg saline	18 hours
Experiment 2			
1	2	8 x 40 mg/kg MDMA**	120 days
2	2	8 x 10 ml/kg saline **	120 days

* From first dose.
** Twice daily for four days.

and wash-steps throughout each staining procedure. A staining basket is easily constructed by cutting off the bottom of a plastic tissue culture dish with a band saw and applying nylon window screen with cyanoacrylate cement ("Super-Glue") or similar. The plastic containers in which the culture dishes are sealed can be used as convenient trays for incubations, washes, and staining steps.

For immunohistochemical stains, primary polyclonal antibodies directed against 5-HT (Incstar Corp., Stillwater, Minnesota) or tyrosine hydroxylase (Eugene Tech. Intl., Allendale, New Jersey) were employed in the present study. Standard primary antibody dilutions of 1:500 in an antibody diluent consisting of 2% normal goat serum, 0.3% Triton-X100, 0.02% sodium azide, and 0.9% sodium chloride are stored in glass scintillation vials at 5°C and can be saved and re-used for up to three years as previously reported (Sofroniew and Schrell, 1982). Prior to use, enough pooled antisera is vortexed and aliquoted in 1.0 ml volumes to numbered scintillation vials to stain the desired number of sections (up to three per vial). In this way, adjacent sections can be incubated in a series of up to 12 vials each containing a different primary antibody in order to determine the relative distribution of the corresponding antigens (e.g.,TH 5-HT, met-enkephalin). Alternately, a larger number of sections (up to 24 in 12 vials) can be incubated in aliquots of the same antisera. Following an overnight incubation in primary antibody at 5°C, sections are placed (using a glass transfer pipette with the tip melted into a small hook) into the numbered compartments of the staining basket while it is immersed in a tray of 0.1 M phosphate buffer. Following three 5-minute changes of buffer, the basket is placed into a secondary antibody (goat anti-rabbit IgG, diluted 1:100 in antibody diluent, Gibco Laboratories, Grand Island, NY) for one hour at room temperature. After another three 5-minute changes of buffer, the sections are transferred into a 24-well culture dish and incubated for one hour, with each well containing one section in 0.2 ml of peroxidase-antiperoxidase complex (Sternberger-Meyer Immunocytochemicals, Inc., Jarrettsville, Maryland) diluted 1:100 in 0.1 M phosphate buffer. The sections are then returned to the staining basket for another three buffer rinses, and the peroxidase is visualized by enzymatic reaction for about 10 minutes with a solution of 0.05% diaminobenzidine (Sigma Chemical Co., St. Louis, Missouri) in buffer, which is filtered and made 0.1% in hydrogen peroxide just prior to use. Sections are then mounted by drying onto gelatin-coated slides, rehydrated

TABLE 2. Experiment 1: Estimates of Density Per Unit Area of Silver-Impregnated (Degenerating) AxonTerminals in Rats Treated with MDMA (Means ± SEM).

		MDMA		
	Control	1x40 mg/kg (18 hr Sac)	1x80 mg/kg (18 hr Sac)	8x80 mg/k (2 wk Sac)
Number of silver-stained terminals per mm^2 of caudate nucleus	2005.3 ± 186	3819.7 ± 446	4118.9 ± 439	3355.0 ± 242

for 30 seconds in distilled water, counterstained (3 minutes in 0.02% cresyl violet), dehydrated through graded ethanols to xylene, and coverslipped with permount.

The silver-degeneration stain was conducted exactly as described by Nadler and Evenson (1983), but using the staining basket system described above. To confirm that the silver-degeneration method was sensitive to and specific for degenerating neuronal structures, sections from rats treated one week previously with 9 mg/kg of the hippocampal neurotoxin trimethyltin (Paule *et al.*, 1986) were included in Experiment 1 as a positive control. Positive control (trimethyltin-treated) sections included in the batch processed for silver-staining showed numerous degenerating dendrites, cell bodies, and axon terminals in the hippocampus (not shown). This demonstrates a successful procedure and is important to include since silver-impregnation stains can be somewhat variable from batch-to batch.

Quantitative estimates of the density per unit area of certain features such as stained cell bodies, axons, and terminal processes were made by counting their number in 8 randomly chosen, non-overlapping fields-of-view (4 fields from each of 2 sections per rat) examined (x400) by an observer blind to the group identification of the sections. The total number of objects observed divided by the area evaluated in mm^2 of 30 micron thick tissue formed a density estimate for each rat; the group means (n = 2-3) of these estimates and their SEM are reported in Tables 2 and 3.

For neurochemical analyses, rats were killed

TABLE 3. Experiment 2: Estimates of Density Per Unit Area of Serotonin-Immunolabeled Axons in Rats Treated with MDMA (Means ± SEM).

		MDMA
	Control	8x40 mg/kg (4 Mon Sac)
Density of serotonin immunoreactive axons per mm^2 of caudate nucleus	180,100 ± 37,600	232,500 ± 4,400
Density of serotonergic axons per mm^2 of layer 1 dorsomedial cortex	92,000 ± 20,000	64,800 ±10,400
Density of serotonergic axons per mm^2 of hippocampus (stratum lacunosum)	142,800 ± 28,400	82,000 ± 8,400
Density of serotonergic cell bodies per mm^2 of dorsal raphe	41,100 ± 21,500	53,000 ± 7,800

by decapitation and their brains rapidly dissected into frontal cortex, caudate nucleus, hippocampus, hypothalamus, frontal cortex, and brain stem as described elsewhere in more detail (Slikker *et al.*, 1988). Tissues were then immediately frozen on dry ice and stored at -80°C until processed. Neurotransmitters and their acid metabolites were resolved by high-performance liquid chromatography (HPLC) and quantified by electrochemical detection as described (Slikker *et al.*, 1986; 1988).

RESULTS

Silver-impregnated terminals could be observed in the caudate sections of Experiment 1 MDMA-treated rats as spidery, multiprocess fine and varicose fibers generally found in proximity to cell bodies (Fig. 1). Similar fibers could be observed, but less frequently, in the control rats (not shown). Counting the silver-impregnated terminals in MDMA rats revealed a 90% increase in the density of affected terminals 18 hr after a single 40 mg/kg dose, and a 105% increase after 80 mg/kg (Table 2). An additional group of rats that had been treated two weeks before sacrifice with eight doses of 80 mg/kg MDMA also exhibited an enhanced number (67% increase) of silver-stained terminal processes in the caudate nucleus. Abundant fibers immunopositive for tyrosine hydroxylase (dopaminergic) were visible throughout the caudate region (Fig. 2) and in the globus pallidus (not shown), but showed no apparent treatment-related effects. In the absence of pre-treatment with tryptophan, only a few serotonin immunopositive fibers could be visualized in Experiment 1 rats.

In Experiment 2, rats were evaluated four months after an eight-dose regimen of MDMA (Table 1). Neuronal cell bodies immunopositive for serotonin were numerous in the

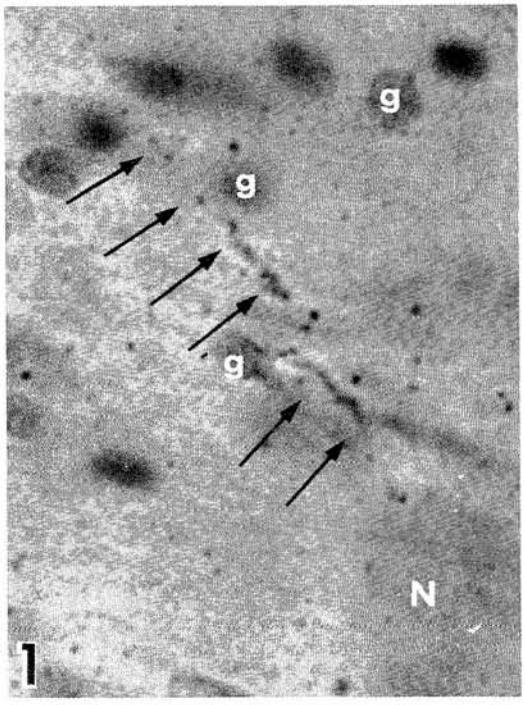

FIG. 1. Arrows indicate a degenerating, silver-impregnated terminal process surrounded by several glia in the caudate nucleus of an MDMA-treated rat (1 x 80 mg/kg, 18 hr sacrifice). G = glia, N = neuronal cell body, x870.

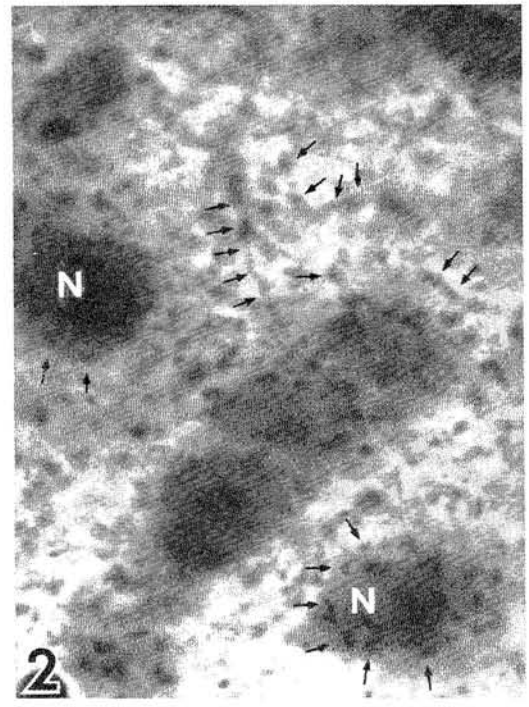

FIG. 2. Tyrosine-hydroxylase-immunoreactive (dopaminergic) axons (small arrows) massively innervate neurons of the caudate nucleus of a control rat. N = neuronal cell-body, x870.

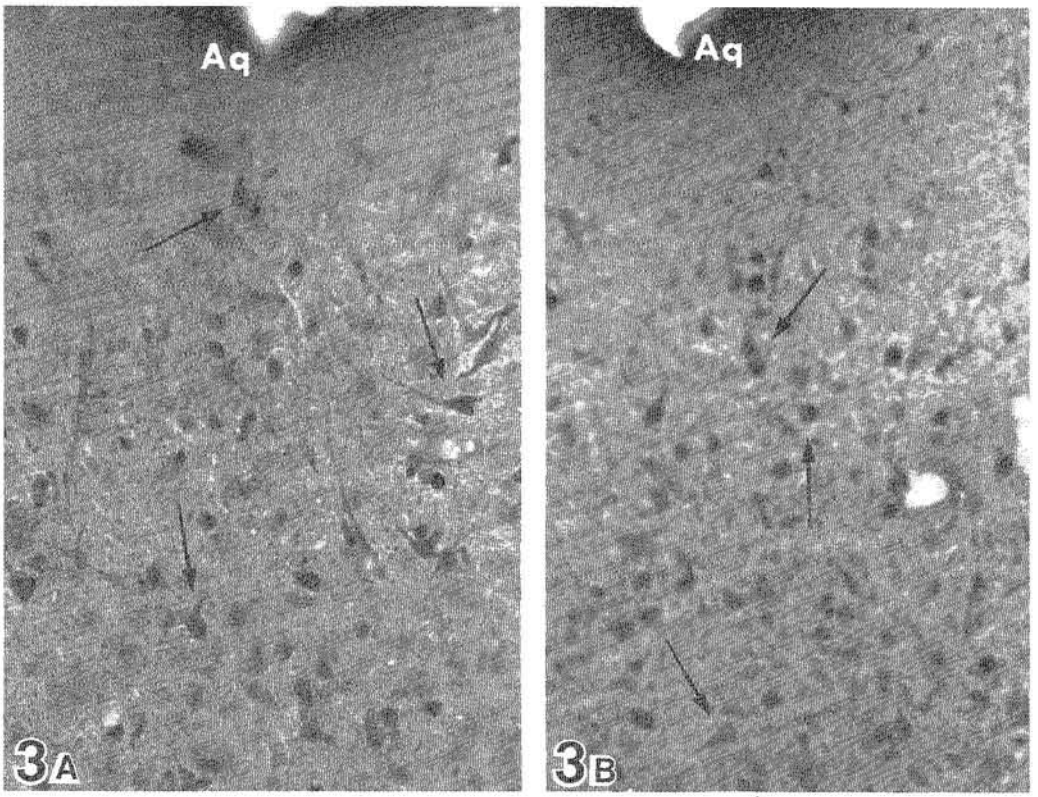

FIG. 3a. Serotonin-containing cell-bodies (arrows) of the dorsal raphe nucleus in the brainstem of a control rat. Aq = ventral extent of the cerebral aqueduct, x 220. **3b.** Serotonin-containing cell-bodies (arrows) were present in similar numbers per unit area in the dorsal raphe nucleus of a rat treated four months prior to sacrifice with MDMA (8 x 40 mg/kg). Aq= ventral extent of the cerebral aqueduct, x 220.

brainstem dorsal raphe nuclei of both treated and untreated rats (Fig. 3a and 3b, Table 3). A number of inclusion bodies could be seen in serotonin immunopositive brainstem cells of treated but not control rats (Fig. 3b). Varicose serotonin-positive fibers similar in size and appearance to the silver-degeneration labeled terminals of Experiment 1 were visible in the caudate nucleus (Fig. 4). Because a long extent of immunostained fiber was being scored in Experiment 2 rather than the discrete terminals of Experiment 1, the density values are not directly comparable between experiments. In the hippocampus, long serotonin-immunoreactive fibers were especially apparent in the stratum lacunosum region among the distal dendritic endings of CA1 pyramidal cells and near the large blood vessels of the hippocampal fissure (not illustrated).

Density estimates of these features revealed that the greatest and most consistent differences observed between treated and control rats was a 43% reduction of 5-HT fibers in the hippocampus of MDMA treated rats. Little difference in numeric density of 5-HT cell bodies between treated and control rats was observed (53,000 vs. 41,100 per mm^2, Table 3). Neurochemical measurement of serotonin content revealed that the most consistent effect of MDMA was a 33% decrement in 5-HT content of the hippocampus ($p < 0.01$ from controls), whereas levels of 5-HT in the brainstem and caudate nucleus were not significantly affected.

DISCUSSION

The results demonstrate that a simple and standard fixation procedure can be used to prepare the same brain for both multiple

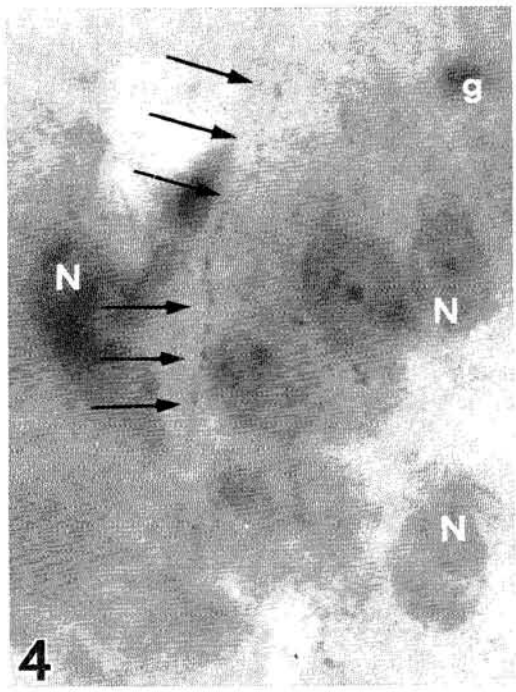

FIG. 4. Arrows indicate a serotonin-immunoreactive axon in relation to Nissl-counterstained cell-bodies in the caudate nucleus of a control rat. Note that the size, number, and appearance of these axons correspond much more closely with the silver-impregnated process of Fig. 1 than do the characteristics of the dopaminergic fibers of Fig. 2. N = neuronal cell-bodies, x870.

antigen immunohistochemistry and a silver-stain specific for degenerating neuronal structures. In Experiment 1, the increased numbers of silver-impregnated (presumed degenerating) fibers counted in the caudate nucleus indicate that MDMA treatment caused degenerative changes as early as 18 hr after a single 40 mg/kg oral treatment. These changes were still apparent up to two weeks following the multiple dose regimen, a time when identically treated cohorts of these animals showed 50-60% decreases of caudate and cortical 5-HT neurochemical content (Slikker *et al.*, 1986; 1988). Schmidt (1987) has reported that the initial decrease (at 3 hr after dosing with 10 or 20 mg/kg MDMA subcutaneously) in 5-HT content was not stereoselective and had recovered by 12-24 hr. The subsequent decrease in neuro-chemical content at 1 week was stereoselective (requiring (+)MDMA) and could be blocked with a serotonin re-uptake inhibitor, fluoxetine, if administered within 6 hr after MDMA. These results could reflect decreased synthesis of serotonin in the absence of morphological damage, but our silver-stain results suggest that even at a time when neurochemical content was unaffected (18 hr, Schmidt, 1987), degenerative changes were present.

Experiment 2 addressed the longevity of the MDMA effects by evaluating rats 4 months following their initial 40 mg/kg oral dose of the series. The most consistent change observed was the notable decrease in density of 5 HT-positive axons in the hippocampus of MDMA rats compared to controls (both groups pre-treated with tryptophan). This finding suggests that at least some of the degenerative changes of Experiment 1 eventually result in the complete loss and removal of hippocampal axons, seen in Experiment 2. This decrease corresponded with the finding that identically treated cohort rats sacrificed concurrently for neurochemistry also showed a decrease in 5-HT content of the hippocampus. The small sample size renders this finding preliminary, but it supports the neurochemical results, which both identify the hippocampus as an area highly sensitive to long-term MDMA effects. The neurohistology also suggests that the neurochemical reductions of 5-HT may correspond to an actual decrease in 5-HT axon number rather than a change in the synthesis rate of serotonin. However, it is important to recognize that such common pharmacological approaches as pre-treatment with monoamine oxidase (MAO) inhibitors or precursor loading (used here) to visualize the 5-HT system anatomically must be cautiously interpreted by the toxicologist. Failure to observe 5-HT-positive fibers can occur due to either their physical absence or to a fiber concentration of 5-HT below the threshold of detectability by immunohistochemical methods. Since Schmidt (1987) described an MDMA-induced loss of serotonin synaptosomal re-uptake sites, it would not be surprising that MAO inhibitors failed to boost a 5-HT signal to detectability in MDMA treated rats; such animals may lack the ability to be enhanced by drug pre-treatments that work by increasing the extracellular 5-HT levels available for re-uptake in the synaptic cleft. More than just immunocytochemical methods are needed to

distinguish this possibility from a situation where the entire 5-HT axonal endings are missing, as is suggested by the silver-degeneration results of Experiment 1. Including immunohistochemical evaluation does help identify the neurochemical contents of the silver-stained neuronal elements; comparing the appearance of micrographs of the caudate nucleus illustrating the degenerating fibers (Fig. 1) to those stained for 5-HT (Fig. 4) or TH (Fig. 2) reveals that the degeneration pattern corresponds much more losely in size, number, and appearance to control micrographs illustrating the serotonergic system in the caudate nucleus, rather than the much denser plexus of dopaminergic innervation of this region.

Another important aspect of the immunohistochemical evaluation is that it revealed clearly that a large population of serotonergic cell-bodies (although perhaps compromised by treatment as indicated by the increased presence of inclusion bodies) remained intact in MDMA-treated rats. These results support the obervation that the brainstem (which contains the cell bodies of origin of all the anterior-projecting 5-HT fibers) was less effected than any other brain area studied in terms of the percent loss of serotonin content in MDMA-treated rats or monkeys (Slikker *et al.*, 1988). Together, our neurohistological and neurochemical findings suggest that the toxicity of MDMA is directed at axonal terminal elements rather than the soma or dendrites of 5HT-containing neurons. Moreover, the lack of prolonged MDMA effects in the caudate nucleus as measured either by neurochemical or immunohistochemical methods indicates recovery or compensation by at least some brain areas.

SUMMARY

1. Treatment of rats with MDMA can produce increased numbers of silver-impregnated (presumably degenerating) axon-terminals as early as 18 hr following a single 40 mg/kg oral dose.

2. The size, number, and appearance of these degenerating terminals is consistent with that of axons and their terminals selectively labeled with antisera to serotonin, but not tyrosine hydroxylase.

3. Cell bodies producing serotonin remained present in large numbers when examined four months after MDMA treatment.

4. A consistent decrease in hippocampal 5-HT fibers four months after MDMA, suggests that at least some of the initially seen degenerating axon terminals may be permanently lost.

5. The combination of a specific stain for neurodegeneration with immunohistochemical evaluation is a vital component, along with neurochemical methods, for the evaluation of neurotoxicants.

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