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Lack of Serotonin Neurotoxicity After Intraraphe Microinjection of (+)-3,4-Methylenedioxymethamphetamine (MDMA)

JOSEPH M. PARIS AND KATHRYN A. CUNNINGHAM[1]

Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77550

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PARIS, J. M. AND K. A. CUNNINGHAM. *Lack of serotonin neurotoxicity after intraraphe microinjection of (+)-3,4-methylenedioxymethamphetamine (MDMA).* BRAIN RES BULL **28**(1) 115–119, 1992.—Systemic administration of 3,4-methylenedioxymethamphetamine (MDMA) produces depletions of serotonin (5-HT) and its primary metabolite, 5-hydroxyindoleacetic acid (5-HIAA), decreases 5-HT reuptake sites and diminishes tryptophan hydroxylase activity in various forebrain regions. MDMA has been shown to be neurotoxic to the fine fibers originating from dorsal raphe (DR) 5-HT neurons but not the beaded fibers from the median raphe (MR) nucleus. In the present experiment, MDMA was microinjected directly into the DR or MR to determine whether differential neurotoxicity developed in the DR versus MR fiber systems as measured by 5-HT levels and immunocytochemistry. Two weeks following stereotaxic injection with either vehicle or (+)MDMA (50 μg base in 2 μl) into the DR or MR, rat brains were assayed for 5-HT and catecholamine content or 5-HT immunocytochemistry. HPLC analysis revealed no significant changes in monoamine or metabolite concentrations in the hippocampus and striatum of rats administered intra-DR or -MR (+)MDMA. Raphe sections stained for 5-HT also did not reveal any apparent neurotoxicity. A single cerebral injection of (+)MDMA does not produce neurotoxicity to 5-HT neuronal systems originating in the raphe, although neurotoxicity of multiple MDMA injections into these raphe nuclei cannot be ruled out.

3,4-Methylenedioxymethamphetamine (MDMA) Serotonin Dorsal raphe Median raphe Neurotoxicity Microinjections Rat

CONSIDERABLE biochemical and immunocytochemical evidence has demonstrated that 3,4-methylenedioxymethamphetamine (MDMA) and its related congeners are selective serotonin (5-HT) neurotoxins. Biochemically, measures of 5-HT function indicate that MDMA markedly reduces the concentrations of 5-HT and its primary metabolite, 5-hydroxyindoleacetic acid (5-HIAA), and depresses the activity of the biosynthetic enzyme tryptophan hydroxylase (TPH) in 5-HT nerve terminal regions including the hippocampus, neostriatum and cerebral cortex in rodents as well as nonhuman primates (16,25). The number of 5-HT uptake sites as measured by [^{3}H]paroxetine binding in frontal cortex is also significantly reduced following MDMA treatment (4). The severity of these neurochemical and neurotoxic effects depend upon the dose utilized, the route of administration as well as the duration of treatment and the posttreatment interval. For example, a single dose (10–20 mg/kg, SC) of MDMA dramatically reduces whole-brain and cortical levels of 5-HT, [^{3}H]-5-HT uptake and TPH activity within three hours (17,18). A subsequent recovery at 6–24 hours gives way to persistent 5-HT dysfunction which lasts for weeks even after a single dose (17,18). Multiple systemic injections of similar doses have been shown to result in a loss of immunohistochemically labelled 5-HT terminals (but not cell bodies), which appears to be specific for the fine axons which emanate from the dorsal raphe (DR) nucleus of the midbrain; the beaded axons of 5-HT neurons from the median raphe (MR) nucleus are spared (14,26).

The effects of MDMA appear to depend upon uptake into 5-HT neurons, since specific 5-HT uptake blockers such as fluoxetine and citalopram prevent the biochemical, electrophysiological and neurotoxic effects of MDMA (3, 4, 17, 23). The midbrain raphe nuclei, which provide 5-HT innervation to the entire forebrain, have high concentrations of 5-HT uptake sites (9) and thus may be a target for the action of MDMA. Indeed, direct application of MDMA onto the midbrain raphe in vitro increases 5-HT release leading to a subsequent diminution of cellular activity (23). However, intracerebroventricular (ICV) or intra-DR injection of (±)MDMA failed to result in alterations in cortical or striatal 5-HT levels or TPH activity when measured 3 hours post-MDMA (19). Thus, direct raphe application of MDMA appears to result in short-term neurochemical, but not neurotoxic, effects on 5-HT systems.

The purpose of the present study was to determine if intraraphe infusion of (+)MDMA results in long-term neurotoxicity when assessed two weeks after injection. A single dose (50 μg) was injected into either the DR or MR. This dose was chosen based upon our unpublished research in which microinjections

[1]Requests for reprints should be addressed to Kathryn A. Cunningham, Ph.D., Department of Pharmacology and Toxicology (J-31), University of Texas Medical Branch, Galveston, TX 77550.

TABLE 1

MONOAMINES IN INTRARAPHE MDMA-TREATED RATS

	pmol/mg Protein					
	5-HT	5-HIAA	DA	DOPAC	NE	HVA
Striatum						
DR	39.8 ± 1.5	36.8 ± 1.6	516.6 ± 46.2	54.1 ± 11.2	28.6 ± 1.7	30.9 ± 3.6
MR	42.5 ± 2.8	37.9 ± 3.0	450.7 ± 19.9	41.8 ± 2.3	24.3 ± 2.8	26.3 ± 3.5
VEH	41.0 ± 1.2	40.3 ± 1.5	421.8 ± 27.2	44.3 ± 2.7	31.8 ± 6.6	27.2 ± 1.9
Hippocampus						
DR	24.1 ± 1.5	21.1 ± 0.7	ND	ND	17.4 ± 1.3	11.6 ± 0.6
MR	23.5 ± 1.4	22.0 ± 1.8	ND	ND	14.9 ± 1.2	11.2 ± 1.4
VEH	20.0 ± 1.2	19.5 ± 1.3	ND	ND	15.4 ± 1.3	12.4 ± 1.6

Content of 5-HT, 5-HIAA, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE) and homovanillic acid (HVA) in rats injected with MDMA (50 μg/2 μl) or ascorbic acid vehicle into either the MR or DR. Data represent mean ± S.E.M. of N = 6–8 subjects/group. Since an initial analysis indicated that there was no difference ($p>0.05$) between the two groups, data from rats that received intra-DR and MR infusions of vehicle were combined.

of other stimulants have been investigated. Although 50 μg is approximately 100–400 times less than the toxic doses used systemically (4,22), this dose of (+)MDMA was chosen as the highest dose not expected to produce *nonspecific* tissue damage. Toxicity to 5-HT axon terminals was assessed by high-performance liquid chromatography (HPLC) analysis of 5-HT and metabolite content in hippocampal and neostriatal tissue, which receive the majority of their 5-HT innervation from the MR and DR, respectively (11). Tissue levels of catecholamines were measured to assess potential toxic effects of intraraphe MDMA microinjection on these neurotransmitters. Although 5-HT cell bodies appear to be unaffected by MDMA, Ali et al. (1) and Ricaurte et al. (16) have recently reported the presence of MDMA-induced inclusion bodies in DR 5-HT perikarya; thus, immunocytochemistry for 5-HT in midbrain tissue slices was used to determine potential neurotoxicity to 5-HT perikarya.

METHOD

Surgery

Male Sprague-Dawley rats (N = 43) were anesthetized with sodium pentobarbital (60 mg/kg, IP). A 30-gauge needle attached to a 10-μl syringe was stereotaxically lowered into the midbrain at the following coordinates: DR (AP, bregma −7.6 mm; ML, −3.2 mm; DV, −6.5 mm; at an angle 25° lateral to the midline); MR (AP, bregma −7.6 mm, ML, −3.6 mm; DV, −7.8 mm; at an angle 25° lateral to the midline) (15). Infusions of (+)MDMA (50-μg base in 2 μl of 0.1% ascorbic acid in saline; NIDA) into DR (N = 15) or MR (N = 15) were made over a 10-min interval. Similarly, control animals were injected with 2 μl of the ascorbic acid vehicle into MR (N = 7) or DR (N = 6). The needle remained in situ for an additional 5 min to allow adequate spread of the solution away from the tip. The animals were monitored daily for postoperative weight changes.

Biochemical Analysis

Two weeks later, the rats (N = 30) were sacrificed by decapitation and the hippocampus and striatum were quickly removed. Tissues were stored at −80°C until monoamine determinations were made. The contents of 5-HT, 5-HIAA, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE) and homovanillic acid (HVA) were concurrently measured in extracts of hippocampus and striatum by an HPLC equipped with a Biophase ODS reverse-phase C_{18} analytical column and an electrochemical detector (Bioanalytical Systems, Lafayette, IN) as previously described (5). Quantities of monoamines and metabolites are expressed as pmol/mg of protein.

5-HT Immunocytochemistry

The remaining rats (N = 13) were deeply anesthetized with sodium pentobarbital (90 mg/kg, IP) and transcardially perfused with 0.1 M calcium-free Tyrodes solution followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline. Sections (32 μm) were cut in a cryostat and subsequently processed for 5-HT immunohistochemistry. A 5-HT antibody made in rabbit (Incstar, Stillwater, MN; 1:25,000–50,000) was used in conjunction with an avidin-biotin peroxidase system (Vector Laboratories, Burlingame, CA). Labeling of 5-HT was visualized with diaminobenzedine (DAB). Adjacent series of tissue sections were stained with cresyl violet acetate. Qualitative assessments of 5-HT staining were made by an investigator blind to the animals' treatment.

Data Analysis

Monoamine levels are expressed as mean ± SEM pmol/mg and were analyzed with an analysis of variance (ANOVA; SAS Institute, Cary, NC). Since an initial analysis indicated that there was no difference ($p>0.05$) between the two groups, data from rats that received intra-DR and -MR infusions of vehicle were combined.

RESULTS

During the postoperative recovery period, adverse behavioral effects such as hyperactivity, which is observed following the nonselective destruction of raphe nuclei by electrolysis or ibotenic acid injection (2,10), were never detected.

Compared to vehicle injection, intra-MR or -DR injection of (+)MDMA two weeks prior to sacrifice did not result in significant alterations in monoamine levels in either the striatum or hippocampus ($p>0.05$ for all amines and metabolites; Table 1). Brainstem coronal sections which included the MR and DR were

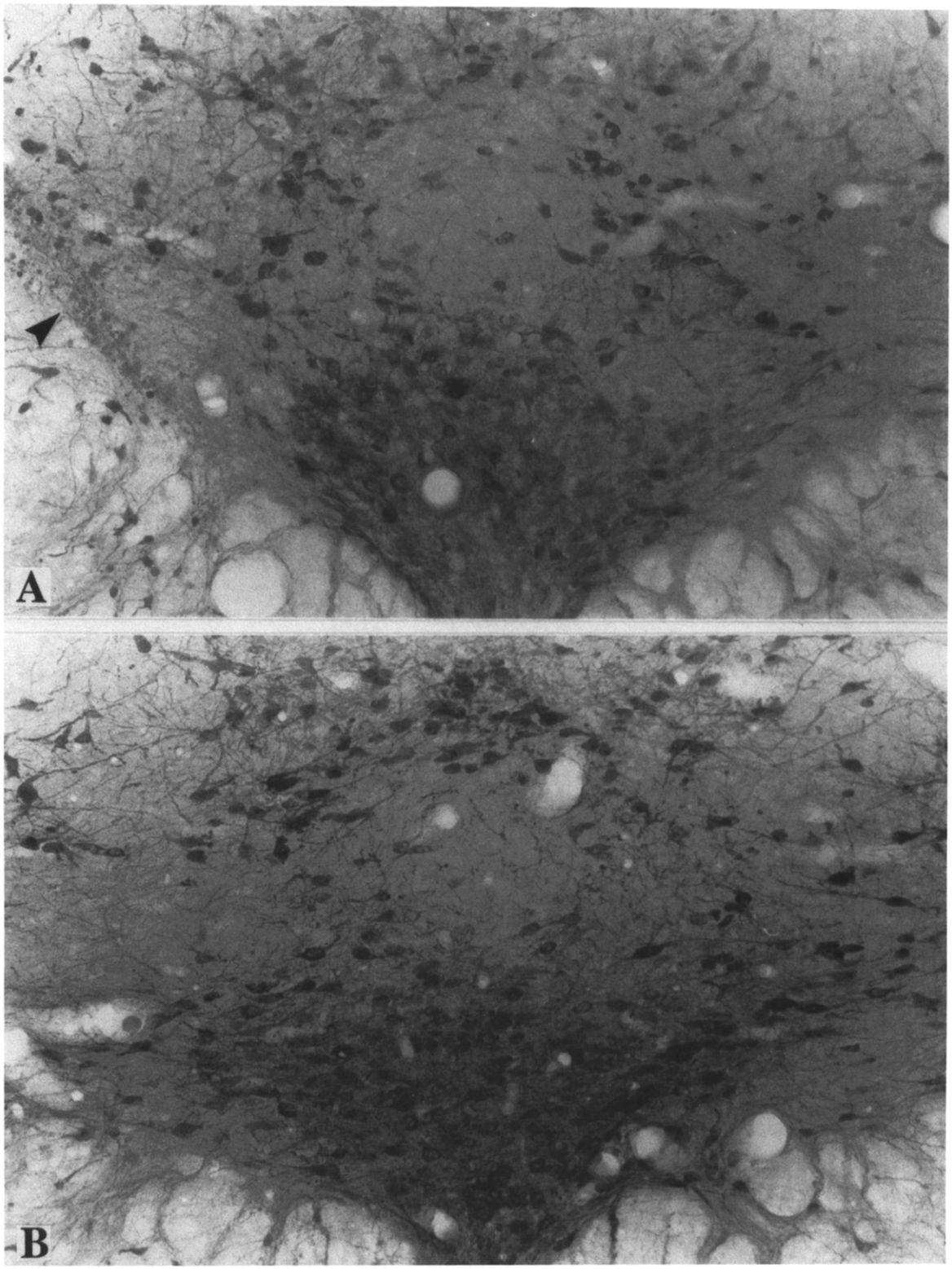

FIG. 1. Coronal sections (32 μm) through the dorsal raphe nucleus (DR) of rats treated with either ascorbic acid vehicle [(A); interaural plane +1.0 mm (15)] or (+)MDMA [50 μg in 2 μl; (B)]. Sections were prepared with 5-HT immunohistochemical procedures. Arrow points to the location of an injection cannula track.

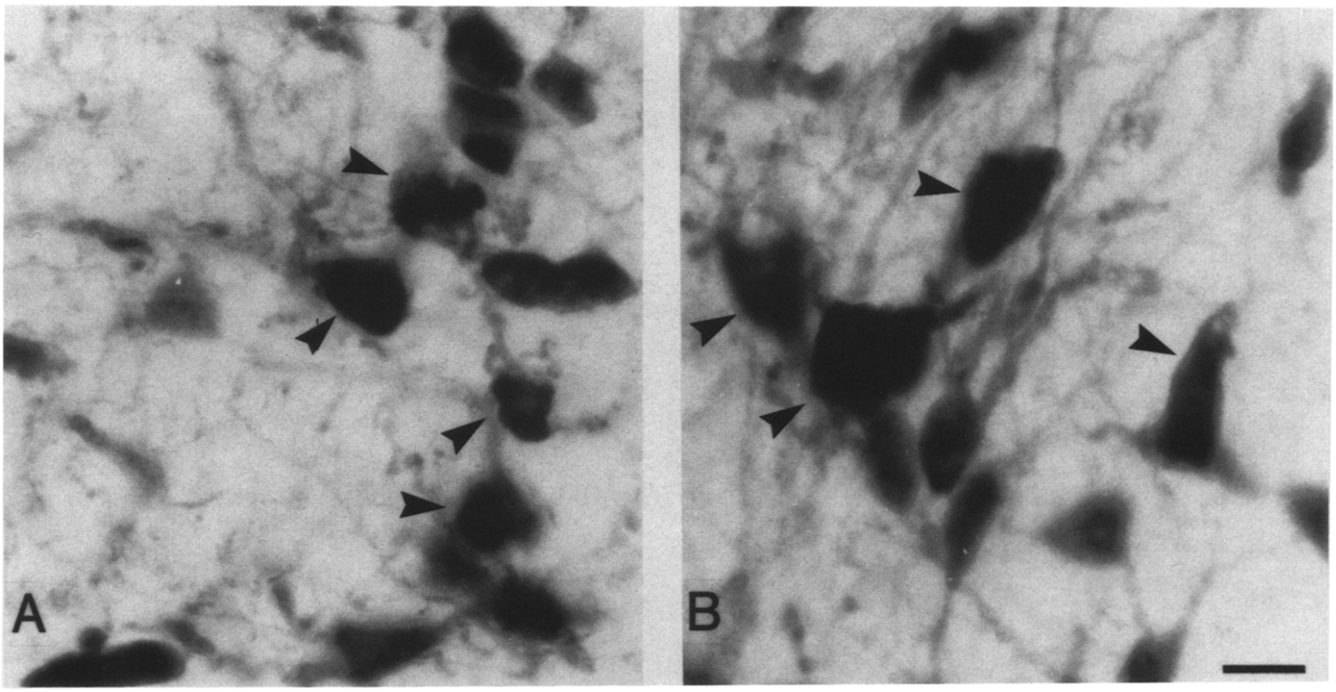

FIG. 2. Magnified view of DR cells stained for 5-HT (arrows) in a vehicle- (A) and intra-DR MDMA-treated (B) rat. Note the lack of any apparent morphological abnormalities. Bar represents 20 μm.

processed immunocytochemically to visualize 5-HT. There was no apparent loss of 5-HT cell bodies or nonspecific damage due to the single (+)MDMA infusion in either the DR or MR (Fig. 1). Blind analysis and closer inspection of the labeled 5-HT neurons in the raphe nuclei (Fig. 2) did not reveal any structural abnormalities such as the presence of inclusion bodies (Fig. 2) which have been observed following systemic administration of (+)MDMA (1,16).

DISCUSSION

Structural analogues of d-amphetamine including MDMA as well as methamphetamine, MDA and fenfluramine induce degeneration of 5-HT axons and terminals when administered systemically (14,21). Our present results suggest that a single microinjection of 50 μg of (+)MDMA into the MR or DR does not result in neurotoxicity to either 5-HT axons/terminals as measured by concentrations of 5-HT and 5-HIAA in hippocampus or striatum. Similarly, after acute (1-h) infusion of relatively large doses of MDMA (2.0 and 6.5 mg/kg, ICV), only minimal alterations in tryptophan hydroxylase activity have been noted (19). Thus, a single intraraphe MDMA injection appears to produce little or no effects on terminal 5-HT neurochemical parameters.

Analysis of midbrain sections stained for 5-HT also suggests that a single intraraphe MDMA injection does not result in amphetamine neurotoxicity. These findings are in agreement with O'Hearn and colleagues (14) who found no observable alterations in 5-HT immunoreactivity and cell morphology in the raphe following systemic MDMA treatment. Interestingly, Ricaurte and co-workers (16) have observed that in nonhuman primates, which appear to be four to eight times more sensitive than the rat to the toxic effects of MDMA, neuropathologic inclusion bodies develop in DR perikarya following systemic exposure to MDMA. These morphological changes occur in the absence of any apparent 5-HT cell loss and were only detectable with hematoxylin/eosin and Luxol Fast Blue-periodic acid Schiff stain (16). Similarly, Ali and co-workers (1) have been able to detect the presence of inclusion bodies in DR 5-HT immunoreactive cells of rats treated with high doses of MDMA (40 mg/kg, b.i.d. for 4 days) and sacrificed 120 days later. These are the only known reports of neuropathological alterations in 5-HT perikarya following systemic MDMA treatment. In the present study, there was no apparent loss of cells or abnormal-appearing perikarya in the 5-HT-immunoreactive tissue (see Figs. 1 and 2). Thus direct application of a single dose of 50 μg (+)MDMA to raphe cell bodies does not appear to induce neurotoxicity. However, pathological changes may be observed following a longer (greater than 2 weeks) survival period (1) or by utilizing other histological staining techniques (16). Alternatively, we cannot rule out the possibility that multiple injections or a higher dose (greater than 50 μg used in this study) of MDMA into these raphe nuclei might prove to be neurotoxic, since it is possible that diffusion away from the site of injection may have precluded enough drug from entering the cells.

Intracerebral MDMA injection may lack neurotoxicity if systemic administration and the subsequent formation of an active metabolite are requirements for neuronal damage. In fact, MDMA microinjection into 5-HT terminal regions such as cerebral cortex also fails to induce neurodegeneration (13), even though cortical regions express 5-HT axonal loss after systemic MDMA (14). One potential neurotoxin candidate is (+)MDA, which is a major metabolite of MDMA and more potent than the parent compound in producing neurotoxicity (8,17). Thus, MDA (or one of *its* metabolites) could be responsible for MDMA-induced neurotoxicity, although there is some evidence against this hypothesis (12). Furthermore, although the molecular mechanisms

are largely unclarified, oxidative products similar to 6-hydroxydopamine, 5,7-dihydroxytryptamine or free radicals may be formed as the result of the excess release and accumulation of DA and 5-HT and could account for amphetamine neurotoxicity (6, 7, 18, 20–22).

The present results indicate that a single intracerebral injection of (+)MDMA into the raphe nuclei does not produce neurochemical or histopathological changes to 5-HT neurons measurable two weeks postinjection. Furthermore, the results demonstrate the need for a better understanding of the molecular mechanisms involved in MDMA-induced toxicity.

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