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Methylenedioxymethamphetamine-Induced Serotonin Deficits Are Followed by Partial Recovery Over a 52-Week Period. Part II: Radioligand binding and autoradiography studies[1]

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ABSTRACT

In our study, age-matched Holtzman Sprague-Dawley rats (275–300 g) received injections with either saline (0.9%) or 3,4-methylenedioxymethamphetamine (MDMA; 20 mg/kg free base, s.c) twice daily for 4 days and allowed to recover for 2, 8, 16, 32 and 52 wk after the final injection before death. Radioligand binding studies with ^{125}I-RTI-55 to dopamine uptake sites in striatal homogenates showed no effect of MDMA on the density of dopamine uptake sites. In contrast, saturation binding studies with ^{125}I-RTI-55 to 5-HT uptake sites in hippocampal and frontal-parietal homogenates showed a significant reduction in the number of uptake sites at 2 wk after MDMA treatment (34 and 25%, respectively of controls). By 16 wk, a partial recovery in the number of 5-HT uptake sites was observed in both tissues; however, only a full recovery of serotonin uptake sites was observed in hippocampus at the end of 52 wk. In more detailed studies using autoradiography with ^{125}I-RTI-55, recovery of serotonin uptake sites varied from region to region. In particular, recovery of 5-HT uptake sites in cerebral cortex was observed to follow a rostral-caudal gradient. In addition, recovery of 5-HT uptake site in hippocampus also followed a rostral-caudal gradient. Different rates of recovery of 5-HT uptake sites were also observed for cingulate cortex, laterodorsal thalamus and ventromedial hypothalamus. No effect of MDMA was observed over lateral hypothalamus, substantia nigra and ventral tegmental area, or over serotonergic cell bodies such as dorsal raphe and median raphe. In conclusion, our study is consistent with previous studies describing the selective neurotoxicity of MDMA for serotonin neurons and presents evidence showing the rate of recovery of 5-HT uptake sites varies according to region and that recovery of 5-HT uptake sites in neocortex and hippocampus follows a rostral-caudal gradient.

MDMA or "ecstasy" is a ring-substituted derivative of methamphetamine with stimulatory and psychotomimetic properties (Anderson *et al.*, 1978; Braun *et al.*, 1980). In the clinical environment, psychiatrists have reported MDMA to "enhance emotions" and "feelings of empathy" and have suggested MDMA could be used as an adjunct to psychotherapy (Greer and Strassman, 1985). However, studies in nonhuman primates show that MDMA is self-administered, thus it has high abuse potential (Beardsley *et al.*, 1986; Lamb and Griffiths, 1987). Consequently, MDMA has been abused for its psychostimulatory properties (Bost, 1988; Peroutka *et al.*, 1988; Shulgin and Jacob, 1982) and has been listed by the DEA as a schedule I drug (U.S. Department of Justice, DEA, 1985).

Depending on route of administration and dosage, repeated administration of MDMA is neurotoxic (Battaglia *et al.*, 1988b; Commins *et al.*, 1987; Li *et al.*, 1989). In the rat, guinea pig and monkey, repeated MDMA depletes central serotonin stores and decreases the number of serotonin uptake sites by causing neurodegeneration of 5-HT nerve terminals (Battaglia *et al.*, 1988b; Battaglia *et al.*, 1987; Commins *et al.*, 1987; De Souza and Battaglia, 1989; Insel *et al.*, 1989; Kleven *et al.*, 1989; Li *et al.*, 1989; Ricaurte *et al.*, 1988a; Ricaurte *et al.*, 1988b; Scanzello *et al.*, 1993; Slikker *et al.*, 1989; Wilson *et al.*, 1989). In contrast, dopaminergic and noradrenergic neurons are unaffected by MDMA (Battaglia *et al.*, 1988a; Battaglia *et al.*, 1987; Li *et al.*, 1989). Although it is clear that MDMA is selectively neurotoxic to serotonin neurons, one question is whether there is recovery or regeneration of serotonin neurons, and if so, how soon recovery

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ABBREVIATIONS: MDMA, methylenedioxymethamphetamine; MDA, methylenedioxyamphetamine; 5-HT, serotonin; DA, dopamine; 5,6 DHT, 5,6 dihydroxytryptamine; 5,7 DHT, 5,7 dihydroxytryptamine; Fr, frontal cortex; Ob, olfactory bulb; FrP, frontal parietal cortex; Str, striatum; S, septum; LD, laterodorsal thalamus; VmH, ventromedial hypothalamus; LH, lateral hypothalamus; H, hippocampus; DH, dorsal hippocampus; Occ-T, occipital temporal cortex; Oc, occipital cortex; vta, ventral tegmental area; sn, substantia nigra; Dr, dorsal raphe; Mnr, median raphe; PBS, phosphate-buffered saline.

occurs. Studies in nonhuman primates have reported long-lasting neurodegeneration of serotonin neurons after MDMA administration (De Souza and Battaglia, 1989; Insel *et al.*, 1989; Ricaurte *et al.*, 1992). In particular, Ricaurte *et al.* (1992) have reported in squirrel monkeys that there is a partial recovery of serotonin levels and uptake sites at 10 wk after MDMA treatment; however, this is not maintained and by 18 mo, serotonin levels and uptake sites have returned to that observed at 2 wk after treatment. Conversely in rat, there are several reports describing MDMA neurotoxicity to be reversible albeit slowly. Battaglia *et al.* (1988b) and Scanzello *et al.* (1993) have reported full recovery of serotonin uptake sites in rat frontal cortex within 32 to 52 wk after MDMA administration. Furthermore, in rats repeatedly treated with the methylenedioxyamphetamine analog, MDA, recovery of serotonin neurons occurs faster in dorsal frontal cortex than in posterior cortex and that 5-HT axonal recovery may follow a rostral-caudal gradient (Molliver *et al.*, 1990; 1989).

In our study, we have used ^{125}I-RTI-55 in radioligand binding and autoradiography studies to measure the recovery of serotonin and dopamine uptake sites in several rat brain regions at 2, 8, 16, 32 and 52 wk after repeated administration of MDMA (2 × 20 mg/kg s.c., 4 days; total = 160 mg/kg) in age-matched rats. As described in our companion paper, "recovery" refers to the return or restoration of the measure in question to control values and does not necessarily imply normal regeneration or reinnervation patterns. Results show that the density of dopamine uptake sites in striatum was not affected by MDMA. In contrast, the number of serotonin uptake sites in most brain regions was severely reduced by the MDMA treatment. In forebrain regions, the density of 5-HT uptake sites gradually returned to control levels by 16 to 32 wk, whereas in posterior brain regions such as the occipital cortex only a partial recovery was seen at 52 wk. The density of serotonin uptake sites in the dorsal and median raphe was unaffected by MDMA and was not significantly different from age-matched controls. In conclusion, our study demonstrates several brain regions to exhibit different patterns of recovery to MDMA neurotoxicity and is in support of the hypothesis that 5-HT neuronal recovery in cerebral cortex and hippocampus follows a rostral-caudal gradient.

Methods

Materials

^{125}I-RTI-55 (specific activity = 2200 Ci/mmol) was obtained from NEN-Dupont (Wilmington, DE). MDMA HCl was obtained from the National Institute on Drug Abuse (Baltimore, MD). Benztropine mesylate and (±) propranolol was purchased from Research Biochemicals International (Natick, MA) and citalopram HCl was purchased from Lundbeck (Denmark). Unlabeled RTI-55 and RTI-121 were generous gifts received from Drs. J. W. Boja (NIDA; Baltimore, MD) and I. Carroll (RTI; Research Triangle Park, North Carolina). All other chemicals and compounds were purchased from Sigma Chemical Co. (St. Louis, MO) and were of analytical grade.

MDMA Treatment

Male Sprague-Dawley rats (Holtzman Co., Madison, WI) weighing approximately 275 to 300 g were housed in pairs under a 12-hr light/dark cycle at room temperature (23 ± 2°C) with free access to food (Teklad 4% rat diet) and water. For saline and MDMA treatments, age-matched rats received injections s.c. with either 0.9% saline (1 ml/kg) or 20 mg/kg MDMA (free base) twice daily (12 hr apart) for 4 consecutive days (total MDMA = 160 mg/kg) and allowed to recover for periods of 2, 8, 16, 32 and 52 wk. After recovery, saline- and MDMA-treated animals were either killed for radioligand binding studies with ^{125}I-RTI-55 or perfused with PBS for autoradiography studies with ^{125}I-RTI-55.

^{125}I-RTI-55 Binding Studies

Membrane preparation. At the end of each recovery period, saline- and MDMA-treated rats were sacrificed and their brains removed and placed on ice. Striatum, hippocampus and frontal-parietal cortex were dissected from rat brain (Heffner *et al.*, 1980) and frozen and stored in liquid N_2. Tissues from saline- and MDMA-treated animals for each recovery period was removed from storage and homogenates prepared according to Boja *et al.* (1992). Briefly, tissues were suspended in 20 vol ice-cold incubation buffer (0.32 M sucrose and 10 mM sodium phosphate pH 7.4), homogenized using a Brinkman polytron (setting 8, 30 sec) and centrifuged at 40,000 × *g* for 10 min. After centrifugation, supernatants were discarded and the pellets resuspended in ice-cold incubation buffer and the centrifugation process repeated. Pellets were finally resuspended in incubation buffer (pH 7.4) at room temperature (23–25°C). For striatum, the final tissue concentration was 1.0 mg original wet weight/ml, while for somatosensory cortex and hippocampus, a final tissue concentration of 12 mg original wet weight/ml was used.

^{125}I-RTI-55 Binding Assay

Cold saturation studies were performed using ^{125}I-RTI-55 to assess the effect of MDMA on dopamine uptake sites in striatum and serotonin uptake sites in hippocampus and frontal-parietal cortex. Briefly, striatal membranes (4–6 μg protein), cortical and hippocampal membranes (40–50 μg protein), respectively, were incubated with ^{125}I-RTI-55 (20 pM) in the absence and presence of increasing concentrations of unlabeled RTI-55 (0–3 nM; total vol = 2 ml). After 60 min, the incubation was terminated by vacuum filtration through Whatman GF/B filters previously soaked in 0.1% PEI. Filters were washed with 3 × 5 ml of ice-cold incubation buffer and counted for radioactivity in an ICN 4/880 Plus gamma counter (78% efficiency). In studies measuring ^{125}I-RTI-55 binding to dopamine uptake sites, nonspecific binding was defined as the binding remaining in the presence of benztropine (3 μM final concentration). Similarly, in studies measuring the recovery of serotonin uptake sites in hippocampus and frontal-parietal cortex, nonspecific binding was defined by citalopram (0.3 μM final concentration). Determination of the number of dopamine and serotonin uptake sites (*i.e.*, B_{max}) and affinity (K_d) from saturation studies was performed by Scatchard analysis using the EBDA and LIGAND computer radioligand data analysis software programs (BIOSOFT, UK).

Autoradiography Studies

Preparation of tissue sections. After each recovery period, saline- and MDMA-treated animals were whole-body perfused with phosphate-buffered saline, pH 7.4, 25°C (composition in mM:NaCl: 136.9; KCl:2.7; Na_2HPO_4:10.0; KH_2PO_4:1.76) at a rate of 20 ml/min for 12 min. Afterward, brains were removed and quickly frozen in isopentane previously cooled in dry ice (−40°C) and stored desiccated at −80°C until tissue sectioning. Tissue sections (10 μm) of saline- and MDMA-treated brains were cut in a Jung cryostat at −20°C, thaw mounted onto subbed microscope slides and stored with desiccant at −20°C until the day of experiment.

^{125}I-RTI-55 autoradiography. On the day of experiment, slide mounted tissue sections were removed from storage and thawed to room temperature. Tissue sections were incubated with ^{125}I-RTI-55 (40–45 pM) in pH 7.4, for 120 min at room temperature (23–25°C). To identify binding only to serotonin uptake sites, the selective dopamine uptake inhibitor RTI-121 (100 nM) was included in the incu-

bation to block ^{125}I-RTI-55 binding to dopamine uptake sites. In addition (±) propranolol (10 μM) was also added to prevent ^{125}I-RTI-55 binding to lipid sites. After incubation, sections were briefly rinsed in PBS (2–3 sec) and washed for 2 × 15 min periods in PBS. Sections were finally rinsed in dH_2O and dried under a stream of cool, dry (desiccated) air. Tissue sections were then placed in spring loaded x-ray cassettes and apposed to x-ray film (Kodak) for 8 to 10 days. Films were photographically developed and fixed using Dektol D19 developer and Rapid Fixer. Autoradiograms were analyzed using the NIH Image 1.51 software program. Quantitation of brain images was performed using ^{125}I-microscales (1.25–640 nCi/mg tissue equivalent) purchased from Amersham Corp., Arlington Heights, IL. Brain regions were identified using cresyl violet histochemical staining in conjunction with the rat stereotaxic atlas of Paxinos and Watson (1986).

Statistical analysis. Statistical analysis of the density of binding sites in brain regions from saline- and MDMA-treated animals (N = 3 for each treatment group and time point) was performed using a two-way ANOVA and *posthoc* Duncan test; differences between saline and MDMA group means were considered significant when $P < .05$ was observed.

Results

Radioligand Binding Studies with ^{125}I-RTI-55

Effect of repeated MDMA administration on dopamine uptake sites. Preliminary experiments (data not shown) demonstrated ^{125}I-RTI-55 binding in striatum was to a single class of sites with the pharmacological characteristics of the dopamine transporter. As summarized in table 1, ^{125}I-RTI-55 binding to dopamine uptake sites in striatal homogenates from age-matched saline- and MDMA-treated animals were not significantly different with respect to affinity and density at each recovery period. The lack of an effect by MDMA on dopamine uptake sites in striatum is consistent with previous observations of dopamine neurons being insensitive to the neurodegenerative effects of MDMA (Battaglia *et al.*, 1988b; Battaglia *et al.*, 1987; Li *et al.*, 1989).

Effect of repeated MDMA administration on serotonin uptake sites. Preliminary experiments (data not shown) demonstrated ^{125}I-RTI-55 binding in frontal-parietal cortex and hippocampus was to a single class of sites with the pharmacological properties of the serotonin transporter.

Saturation studies were performed with ^{125}I-RTI-55 to measure the density of serotonin uptake sites in tissues from saline and MDMA treated animals. As summarized in table 1, ^{125}I-RTI-55 bound to 5-HT uptake sites in saline- and MDMA-treated tissues with high affinity. However, there was a significant difference in the number of serotonin uptake sites in hippocampus and frontal-parietal cortex from MDMA-treated animals compared with that from age-matched saline-treated animals. In hippocampus, the density of 5-HT uptake sites was significantly reduced to 34% of control at 2 wk after MDMA treatment. Sixteen weeks after MDMA treatment, the density of 5-HT uptake sites in hippocampus from MDMA-treated animals was still significantly reduced compared to that from saline-treated animals. However, statistical analysis showed that the density of 5-HT uptakes in hippocampus at this recovery period was significantly greater than that observed at 2 weeks after MDMA treatment, thus indicating a partial recovery. Further recovery of serotonin uptake sites continued, since at 52 weeks the density of 5-HT uptake sites in the MDMA exposed hippocampus was not significantly different from saline controls (table 1) and was significantly greater than that observed at 16 wks.

In frontal-parietal cortex, repeated administration of MDMA significantly reduced the density of 5-HT uptake sites without affecting binding affinity. At 2 weeks after treatment, the density of 5-HT uptake sites in MDMA treated tissue was 25% of that in age-matched saline treated animals. As in hippocampus, a partial recovery of serotonin uptake sites was observed at 16 wk after MDMA administration. However, recovery did not continue beyond 16 wk

TABLE 1

Effect of repeated administration of MDMA (20 mg/kg s.c twice daily for 4 days) on ^{125}I-RTI-55 binding to dopamine uptake sites in striatum and serotonin uptake sites in hippocampus and frontal-parietal cortex at 2, 8, 16, 32 and 52 wk after MDMA treatment[a]

Tissue Treatment	Striatum		Hippocampus		Frontal-parietal cortex	
	K_d (nM)	B_{max} (pmol/mg prot.)	K_d (nM)	B_{max} (fmol/mg prot.)	K_d (nM)	B_{max} (fmol/mg prot.)
2 wk Saline	0.52 ± 0.08	5.50 ± 0.63	0.16 ± 0.03	311 ± 31	0.11 ± .02	219 ± 23
2 wk MDMA	0.73 ± 0.09	6.40 ± 0.55 (116.3)[b]	0.19 ± 0.03	108 ± 14 (34.68)	0.17 ± .04	54 ± 9 (24.76)
8 wk Saline	0.48 ± .05	5.38 ± 0.65	0.23 ± 0.03	365 ± 31	0.12 ± 0.02	285 ± 23
8 wk MDMA	0.57 ± 0.09	5.15 ± 0.73 (95.7)	0.17 ± 0.03	86 ± 8 (23.6)	0.15 ± 0.03	83 ± 15 (29.0)
16 wk Saline	0.52 ± 0.06	5.64 ± 0.61	0.17 ± 0.03	334 ± 25	0.14 ± 0.03	307 ± 19
16 wk MDMA	0.54 ± 0.09	5.64 ± 1.42 (100)	0.14 ± 0.02	176 ± 10 (52.74)	0.14 ± 0.03	144 ± 19 (47.03)
32 wk Saline	0.53 ± 0.06	4.93 ± 0.36	0.16 ± 0.03	281 ± 25	0.12 ± 0.02	270 ± 16
32 wk MDMA	0.51 ± 0.07	4.48 ± 0.92 (90.87)	0.15 ± 0.03	196 ± 24 (69.81)	0.10 ± 0.02	167 ± 23 (61.90)
52 wk Saline	0.48 ± 0.09	2.97 ± 0.38	0.19 ± 0.04	323 ± 33	0.12 ± 0.02	294 ± 38
52 wk MDMA	0.57 ± 0.11	3.99 ± 0.84 (134.34)	0.20 ± 0.04	279 ± 20 (86.41)	0.14 ± 0.02	175 ± 21 (59.56)

[a] Values shown are mean ± S.E.M.
[b] Values in parentheses are % recovery of age-matched saline controls.

because the density of 5-HT uptake sites in cortical tissue from MDMA treated animals at 32 and 52 wk was not significantly different from that at 16 wk and was still significantly less than their age-matched controls (table 1). These results show that recovery of 5-HT uptake sites from the neurodegenerative effects of MDMA in hippocampus and frontal-parietal cortex occurs at different rates. To determine if recovery of 5-HT uptake sites may vary in other regions, autoradiography with ^{125}I-RTI-55 was performed.

Autoradiography Studies with ^{125}I-RTI-55

Distribution of ^{125}I-RTI-55 binding sites in rat brain. Coronal tissue sections (10 μm) corresponding approximately to Plates 6, 9, 13, 18, 27, 30, 33, 36, 40 and 49 of a rat brain stereotaxic atlas (Paxinos and Watson, 1986) were cut from brains of saline- and MDMA-treated animals and incubated with ^{125}I-RTI-55 (in the presence of RTI-121). After incubation, autoradiograms were prepared, visualized and quantitated as described in "Methods." Figure 1 shows the distribution of serotonin uptake sites in various rat brain regions at 2 wk after treatment with either saline or MDMA. In saline-treated tissues, ^{125}I-RTI-55 binding was localized over various serotonergic terminal fields including frontal, cingulate, frontal-parietal, occipital-temporal and occipital cortex, hippocampus, laterodorsal thalamus, lateral and ventromedial hypothalamus, olfactory bulb and tubercles, nucleus accumbens and caudate (fig. 1). ^{125}I-RTI-55 binding was also localized over substantia nigra, ventral tegmental area and dorsal and median raphe (fig. 1). Furthermore, ^{125}I-RTI-55 binding in these regions was blocked by the 5-HT uptake inhibitor citalopram thereby indicating specificity of ^{125}I-RTI-55 binding for 5-HT uptake sites (not shown). In parallel experiments with MDMA-treated tissues, ^{125}I-RTI-55 binding in terminal field regions was abolished or significantly reduced by MDMA, although binding in substantia nigra, ventral tegmental area, dorsal and median raphe and lateral hypothalamus was unaffected (fig. 1).

In figure 2, the recovery of serotonin uptake sites from 2 to 52 wk in several brain regions parallel to dorsal hippocampus [*i.e.*, Bregma -2.30; PL 27 of Paxinos and Watson (1986)] is shown. ^{125}I-RTI-55 binding in frontal-parietal cortex, dorsal hippocampus, laterodorsal thalamus, ventromedial hypothalamus but not lateral hypothalamus was significantly reduced or abolished by MDMA at 2 wk after treatment. Binding in ventromedial hypothalamus from MDMA-treated tissues was similar to that from saline animals by 16 wk. Furthermore, no significant difference was observed in the ventromedial hypothalamic regions of saline- and MDMA-treated animals at 32 and 52 wk.

Effect of MDMA on serotonin uptake sites in various cortical brain regions. In studies using MDA, Molliver *et al.*, (1990; 1989) reported recovery of serotonin neurons to occur more rapidly in dorsal frontal cortex than in posterior cortex and that recovery may follow a rostral-caudal gradient. Consequently, we have measured the recovery of serotonin uptake sites in several cortical regions (fig. 3). In frontal cortex (fig. 3A; PL 6), serotonin uptake sites were significantly reduced 2 wk after MDMA administration but by 16 wk, a partial recovery was observed and by 32 wk the density of 5-HT uptake sites in MDMA-treated frontal cortex was similar to control values. In subsequent caudal tissue sections of cortex down to the level of the anterior commisure (*i.e.*, fig. 3, B–C; PL 13–18), a similar rate of recovery of serotonin uptake sites was observed. At the level of the dorsal hippocampus (fig. 3D; PL 27) and extending through to the occipital-temporal cortex (fig. 3, E–H; PL 33–40) and occipital cortex (fig. 3I; PL 49), a different pattern of recovery for 5-HT uptake sites in cortex was observed. The density of 5-HT uptake sites in these cortical regions was reduced to at least the same degree by MDMA as that observed in the forebrain cortical regions (fig. 3, A–C; PL 6–18); however, a partial recovery was not observed until 32 wk after MDMA treatment, indicating a slower rate of recovery. Further recovery in these caudal cortical regions was not seen at 52 wk (fig. 3).

The recovery of serotonin uptake sites at several different levels of hippocampus was also examined. Figure 4A shows the recovery of serotonin uptakes in dorsal hippocampus (PL 27) and more caudal regions of hippocampus (plates 33, 36 and 40; fig. 4, B–D). Although the density of serotonin uptake sites at each level of hippocampus was significantly reduced at 2 wk after MDMA treatment, different rates of recovery of serotonin uptake sites were seen at each level of hippocampus examined. In dorsal hippocampus (fig. 4A; PL 27), recovery of 5-HT uptake sites was complete by 16 wk whereas more caudal levels of hippocampus (*i.e.*, fig. 4, B and C; PL 33 and 36) recovery of 5-HT uptake sites did not occur until 32 wk after MDMA administration. In posterior hippocampus (fig. 4D; PL 40), the density of serotonin uptake sites was still significantly reduced compared to age-matched controls at 52 wk after MDMA treatment.

The effect of MDMA and recovery of serotonin uptake sites in several other brain regions was also examined and are shown in figure 5. In cingulate cortex (fig. 5A; PL 13), the density of serotonin uptake sites 2 wk after MDMA treatment was 20% of that in saline-treated animals, but had significantly increased to 43% of control by 8 wk and was completely recovered by 16 wk. Serotonin uptake sites in septum (fig. 5B; PL 18) and ventromedial hypothalamus (fig. 5D; PL 27) also demonstrated a similar pattern of recovery as that in cingulate cortex. In contrast, serotonin uptake sites in laterodorsal thalamus (fig. 5C; PL 27) was significantly reduced throughout each recovery period, although a partial recovery was seen at 52 wk (fig. 5C).

Although the above data show a number of serotonergic regions to be affected by MDMA, several serotonergic regions were also unaffected. The density of serotonin uptake sites in lateral hypothalamus (fig. 6A; PL 30), substantia nigra and ventral tegmental area (fig. 6B; PL 40), dorsal raphe and median raphe (fig. 6C–D; PL 49) in rats treated with MDMA were not significantly different from their age-matched saline controls at each recovery period.

Discussion

In our study, rats were subjected to a regimen of MDMA (20 mg/kg s.c., twice daily for 4 days) and allowed to recover for various periods of 2 to 52 wk. Radioligand and autoradiography studies with ^{125}I-RTI-55 were performed to measure changes in density of either dopamine or serotonin uptake sites in various brain regions. Because dopamine and serotonin uptake sites are located on dopamine and serotonin nerve terminals respectively, changes in the density of uptake sites in terminal field regions can be used as an index for neuronal degeneration (Battaglia *et al.*, 1988b). It should be

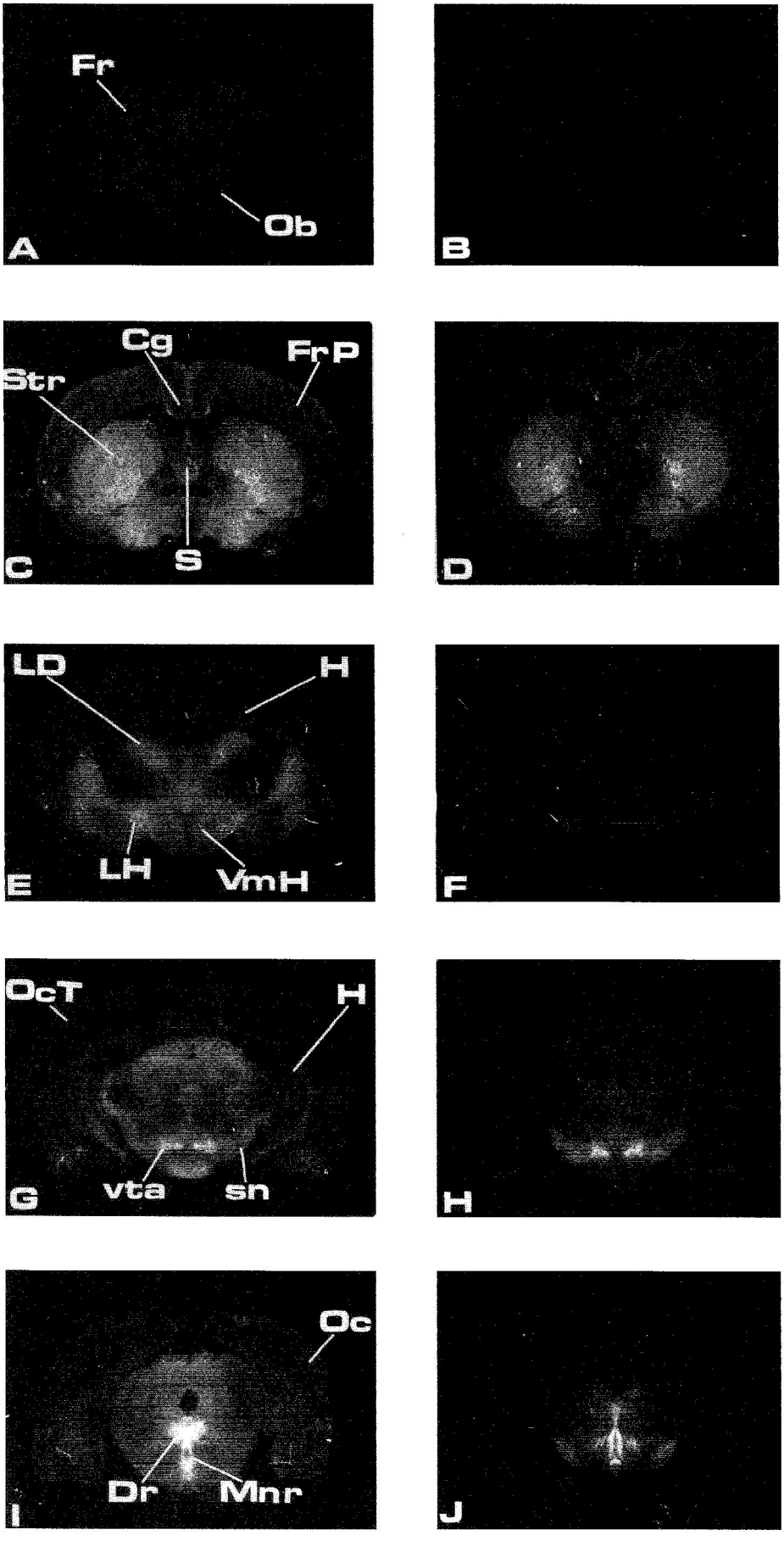

Fig. 1. Distribution of ^{125}I-RTI-55 binding to serotonin uptake sites in various regions of rat brain from rostral to caudal. Left: Distribution of serotonin uptake sites in coronal sections from a saline treated rat at 2 wk after treatment (fig. 1, A, C, E, G and I). Right: Effect of repeated MDMA treatment (20 mg/kg, twice daily for 4 days) on the density of serotonin uptake sites in rat brain 2 wk after MDMA treatment (Fig. 1, B, D, F, H and J). Abbreviations: Fr: frontal cortex; Ob: olfactory bulb; Cg: cingulate cortex; FrP: frontal parietal cortex; Str: striatum; S: septum; LD: laterodorsal thalamus; LH: lateral hypothalamus; VmH: ventromedial hypothalamus; H: hippocampus; Occ-T: occipital-temporal cortex; Oc: occipital cortex; vta: ventral tegmentum area; sn: substantia nigra; Dr: dorsal raphe; Mnr: median raphe. Note: the raphe nuclei shown in the saline group are located rostral of that shown in the MDMA-treated group.

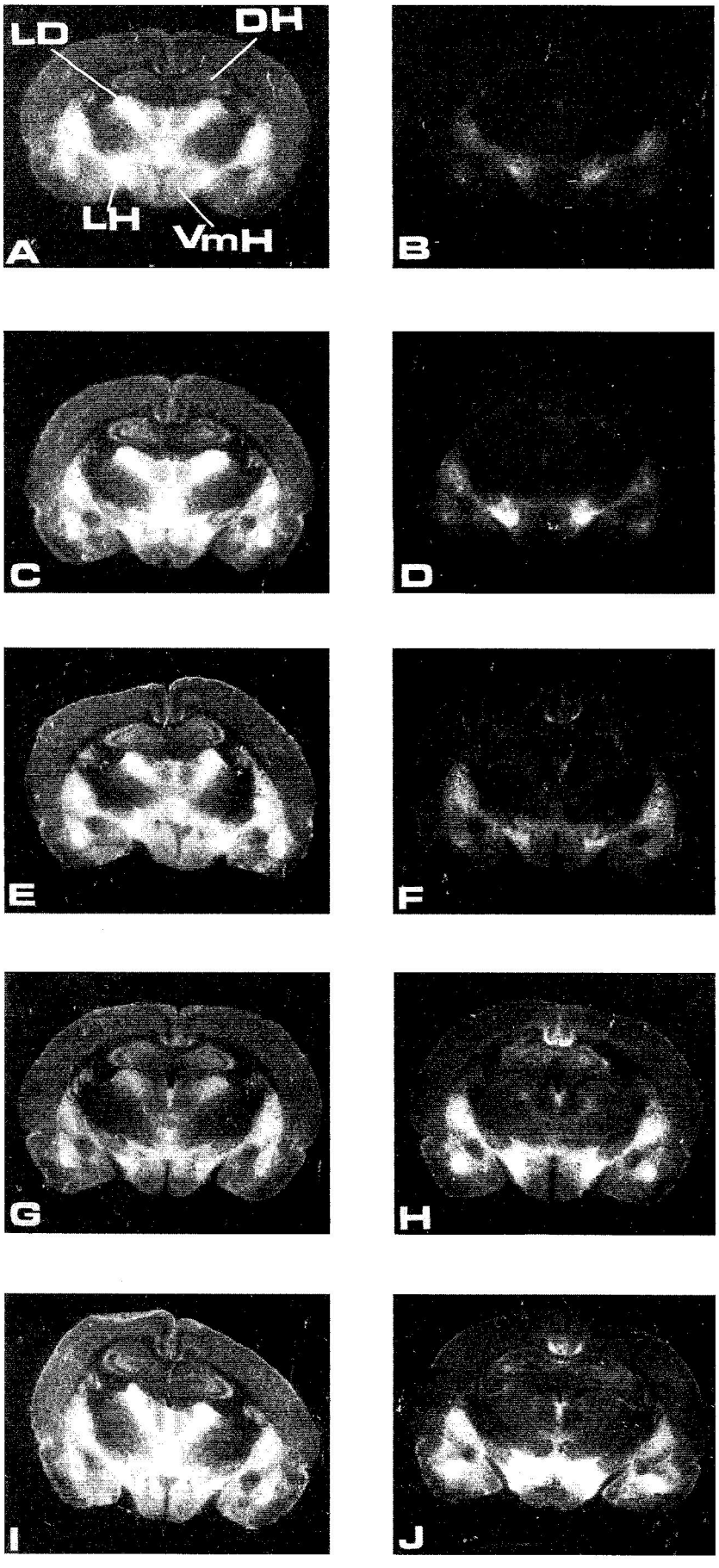

Fig. 2. Effect of MDMA (20 mg/kg s.c; twice daily for 4 days) on the density of serotonin uptake sites in several brain regions at the level of dorsal hippocampus (PL 27). Left: Tissue sections from saline treated animals at 2, 8, 16, 32 and 52 wk (fig. 2, A, C, E, G and I). Right: Tissue sections from MDMA treatment (20 mg/kg, twice daily for four days) animals at 2, 8, 16, 32 and 52 wk (fig. 3, B, D, F, H and J). Abbreviations: LD: laterodorsal thalamus; LH: lateral hypothalamus; VmH: ventromedial hypothalamus; DH: dorsal hippocampus. Recovery of serotonin uptake sites was measured by ^{125}I-RTI-55 binding to 5-HT uptake sites at 2, 8, 16, 32 and 52 wk.

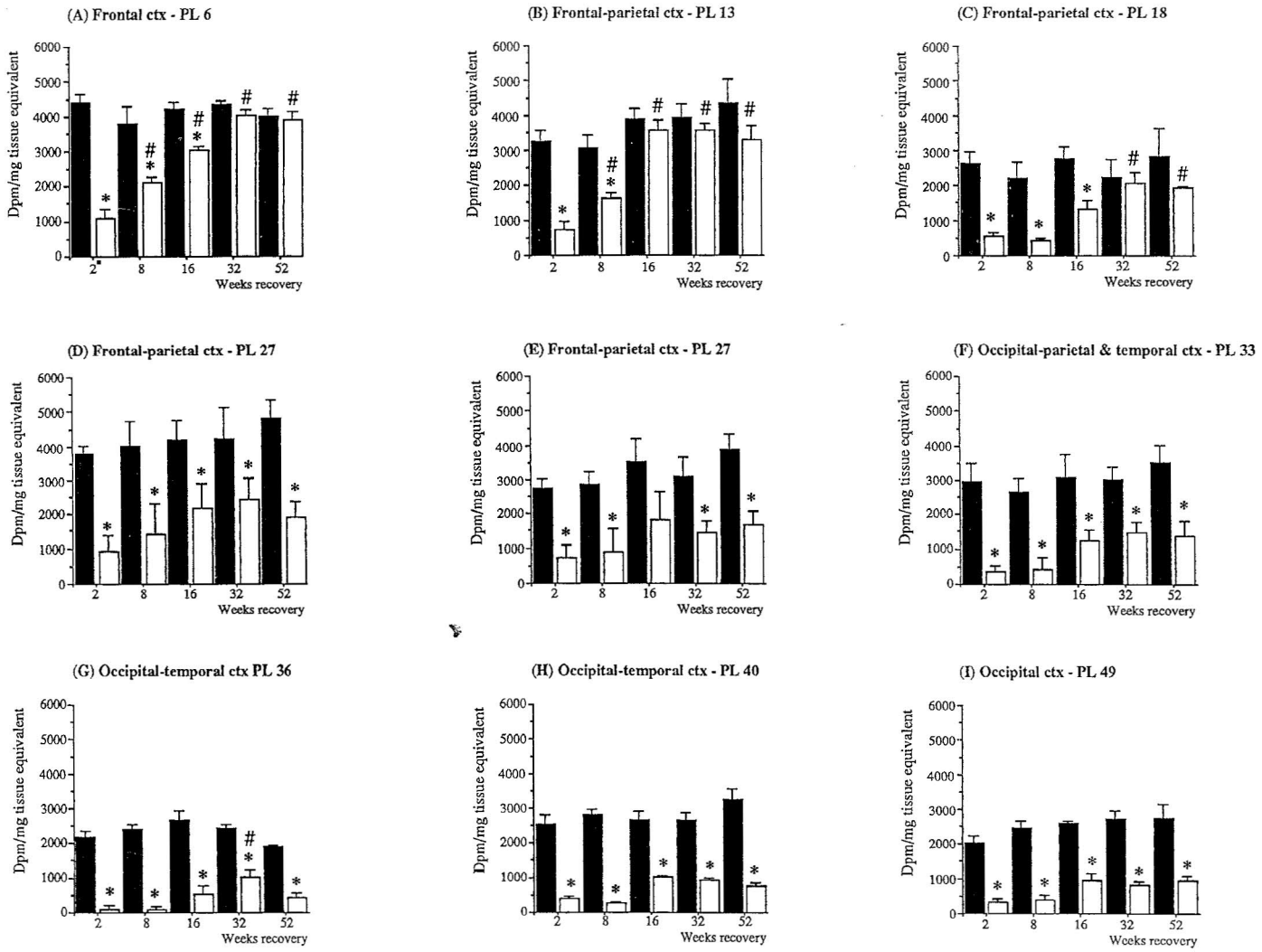

Fig. 3. Effect of MDMA treatment (20 mg/kg s.c twice daily for 4 days) on serotonin uptake sites at various levels of cerebral cortex from rostral to caudal. (A) Frontal cortex—PL6; (B) frontal-parietal ctx—PL13; (C) frontal-parietal cortex—PL18; (D) frontal-parietal cortex—PL27; (E) frontal-parietal cortex—PL30; (F) occipital-parietal and temporal cortex PL33; (G) occipital-temporal cortex—PL36; (H) occipital-temporal cortex—PL40 (I); occipital cortex—PL49. [plate nos. were taken from Paxinos and Watson (1986)]. Values shown are means ± S.E.M. of observations of two to four adjacent tissue sections in individual rats with $n = 3$ for each saline and MDMA treatment and recovery group. Statistical analysis was performed using a two-way ANOVA followed by a *posthoc* Duncan's test. Differences between saline- and MDMA-treated animals were significant when $P < .05$ was observed. *significantly different from age-matched saline; #significantly different from 2 wk MDMA; saline (■) and MDMA (□).

noted that a decrease in the density of neuronal uptake sites as measured by radioligand binding does not by itself imply neuronal degeneration because several factors such as allosteric changes in the actual binding unit of the transporter protein could also result in decreased binding. However, in the companion paper (Sabol *et al.*, 1996) central serotonin levels and active uptake of serotonin into hippocampal synaptosomes were also reduced by MDMA treatment although central dopamine levels and dopamine uptake were unaffected. Consequently, it can be concluded that the observed decrease in ^{125}I-RTI-55 binding to 5-HT uptake sites was due to degeneration of serotonin axons induced by MDMA. These results are in agreement with previous reports describing the selective neurodegenerative effects of MDMA on serotonin neurons (Battaglia *et al.*, 1988b; Battaglia *et al.*, 1991; Battaglia *et al.*, 1987; Commins *et al.*, 1987; DeSouza and Battaglia, 1989; Li *et al.*, 1989; Molliver *et al.*, 1990; Molliver *et al.*, 1989; Ricaurte *et al.*, 1988a; Ricaurte *et al.*, 1988b; Ricaurte *et al.*, 1993; Ricaurte *et al.*, 1992; Scanzello *et al.*, 1993; Sharkey *et al.*, 1991).

Although loss of uptake sites and neurotransmitter is indicative of neuronal degeneration, it must be realized that restoration of these measures do not necessarily imply normal axonal or neuronal regeneration and therefore normal behavioral recovery. In studies with 5,6-DHT, Bjorklund *et al.* (1973) observed abnormal reinnervation patterns of 5-HT axons coinciding with the return of ^{3}H-5HT uptake. In view of this, we have used "recovery" in this and the companion paper (Sabol *et al.*, 1996) to describe the return or restoration of 5-HT levels and uptake sites to saline control values.

Throughout the course of these studies, the MDMA-induced decrease in 5-HT uptake sites in terminal field regions was similar from region to region (*i.e.*, 75–85% reduction) thus implying that 5-HT axons in terminal regions such as cortex and hippocampus are equally sensitive to MDMA. This conflicts with several immunohistochemical studies de-

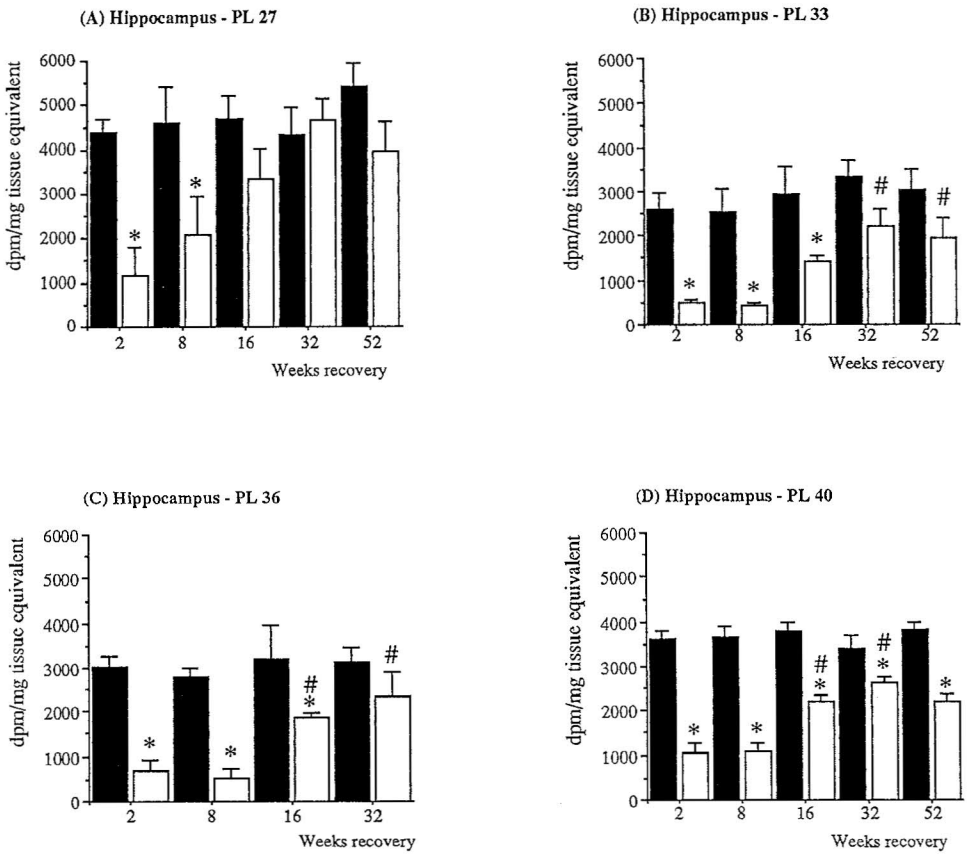

Fig. 4. Effect of MDMA treatment (20 mg/kg s.c twice daily for 4 days) on serotonin uptake sites in hippocampus at various levels from rostral to caudal. (A) Dorsal hippocampus (PL27); (B) hippocampus at the level of lateroposterior thalamus (PL33); (C) hippocampus, anterior to substantia nigra and vta (PL36); (D) posterior hippocampus [PL40; PL nos. were taken from Paxinos and Watson (1986)]. Values shown are means ± S.E.M. of observations of two to four adjacent tissue sections in individual rats with $n = 3$ for each saline and MDMA treatment and recovery group. Statistical analysis was performed using a two-way ANOVA followed by a *posthoc* Duncan's test. Differences between saline- and MDMA-treated animals were significant when $P < .05$ was observed. *significantly different from age-matched saline; #significantly different from 2 wk MDMA; saline (■) and MDMA (□).

scribing the existence of two anatomically distinct classes of serotonin neurons with different neurotoxic sensitivity to MDA, PCA and MDMA (Mamounas and Molliver, 1988; Mamounas *et al.*, 1991; O'Hearn *et al.*, 1988; Wilson *et al.*, 1989). In such studies, "fine" serotonin neurons that arise from dorsal raphe cell bodies have been reported to be vulnerable to PCA, MDA and MDMA, although "beaded" serotonin neurons from median raphe cell bodies are insensitive to the neurotoxic effects of these amphetamine analogs. Furthermore the distribution of fine and beaded 5-HT neurons in

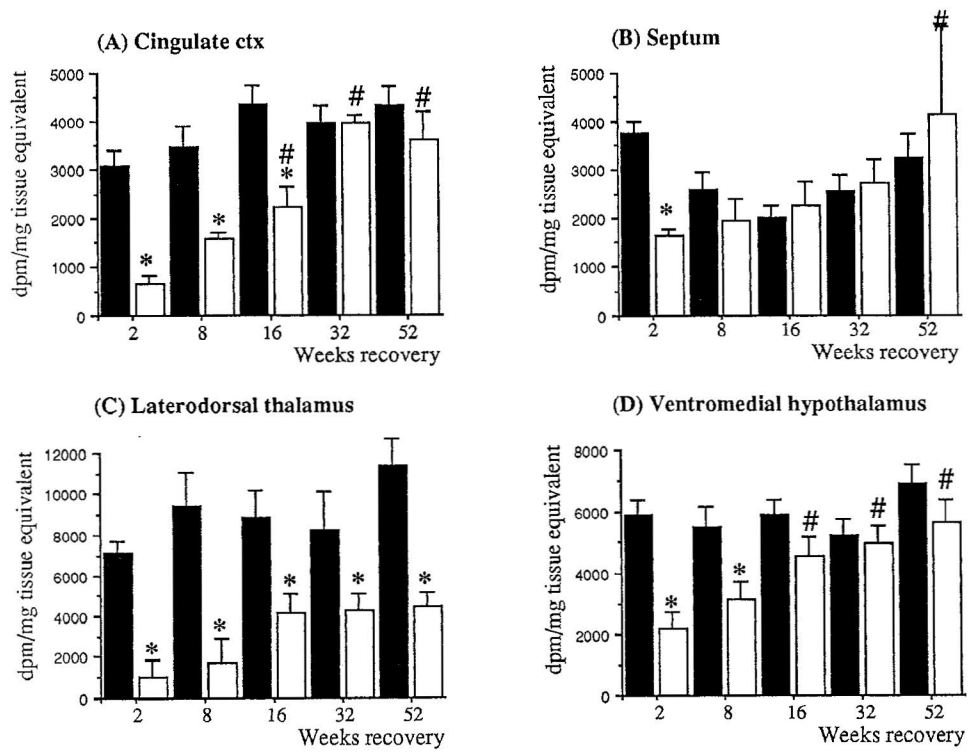

Fig. 5. Effect of MDMA treatment (20 mg/kg s.c twice daily for 4 days) on serotonin uptake sites in various brain regions. (A) Cingulate cortex; (B) septum; (C) laterodorsal thalamus; (D) ventromedial hypothalamus. Values shown are means ± S.E.M. of observations of two to four adjacent tissue sections in individual rats with $n = 3$ for each saline and MDMA treatment and recovery group. Statistical analysis was performed using a two-way ANOVA followed by a *posthoc* Duncan's test. Differences between saline- and MDMA-treated animals were significant when $P < .05$ was observed. *significantly different from age-matched saline; #significantly different from 2 wk MDMA; saline (■) and MDMA (□).

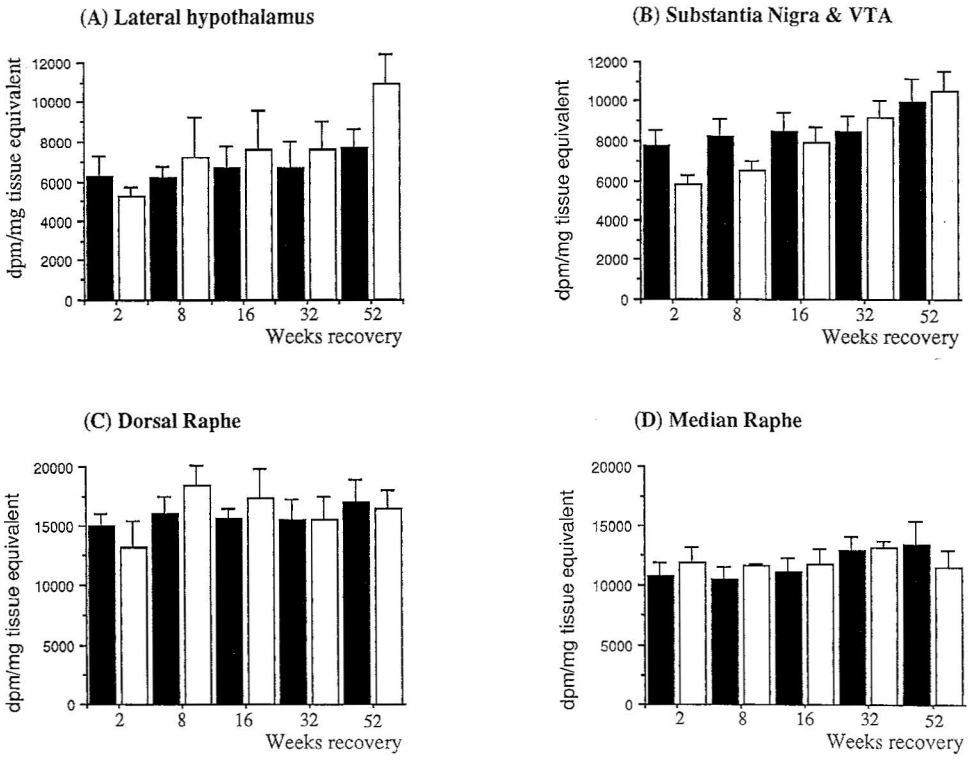

Fig. 6. Effect of MDMA treatment (20 mg/kg s.c twice daily for 4 days) on serotonin uptake sites (A) Lateral hypothalamus (B) substantia nigra and vta; (C) dorsal raphe; (D) median raphe. Values shown are means ± S.E.M. of observations of two to four adjacent tissue sections in individual rats with $n = 3$ for each saline and MDMA treatment and recovery group. Statistical analysis was performed using a two-way ANOVA followed by a *posthoc* Duncan's test. Differences between saline- and MDMA-treated animals were significant when $P < .05$ was observed. *significantly different from age-matched saline; #significantly different from 2 wk MDMA; saline (■) and MDMA (□).

various brain regions is discrete (Kosofsky and Molliver, 1987; Mamounas and Molliver, 1988; Molliver, 1987). Accordingly, innervation of cerebral cortex by fine serotonin neurons is greatest in frontal cortex and is least in posterior cortical regions such as occipital cortex. Conversely, beaded serotonin axons are almost absent from frontal cortex and are present in areas such as occipital cortex. In addition, beaded 5-HT axons are relatively more abundant in hippocampus than in cortex. However, in our study at 2 wk after treatment, the loss of 5-HT uptake sites in rostral cortical regions (76% decrease in frontal cortex) was less than that in posterior or caudal cortical regions (84% decrease in occipital cortex) and thus contradicts the contention of the existence of MDMA-sensitive and -insensitive serotonin neurons because a greater loss of 5-HT uptake sites should have been observed in rostral cortex. Also the loss of serotonin uptake sites in frontal cortex (76% reduction) and dorsal hippocampus (73% reduction) were similar whereas according to the hypothesis there should be very little effect of MDMA in dorsal hippocampus compared to frontal cortex. In the companion paper (Sabol *et al.*, 1996) 5-HT tissue concentrations in frontal cortex and hippocampus were reduced by 70 and 60%, respectively, after MDMA treatment, thus again demonstrating 5-HT axons in both regions to be sensitive to MDMA. At present, there is no apparent explanation for the discrepancy, although Battaglia *et al.* (1991) using ^{3}H-paroxetine to bind to 5-HT uptake sites have reported similar observations of a single class of 5-HT neurons being sensitive to MDMA.

A partial recovery of serotonin uptake sites in cerebral cortex was first observed in frontal cortex at 8 wk after MDMA treatment and was complete by 32 wk. Conversely, a partial recovery of serotonin uptake sites in occipital cortex was not observed until 32 wk after MDMA treatment. This temporal recovery pattern of serotonin uptake sites in cortex is in good agreement with several immunohistochemical studies describing the temporal recovery of serotonin axons in the cerebral cortex of rats treated with either methylendioxyamphetamine or p-chloroamphetamine (Molliver *et al.*, 1989). In such studies, approximately 4 to 8 wk after either methylendioxyamphetamine or p-chloroamphetamine treatment, axons originating from the dorsal raphe appear in the dorsal frontal cortex. These axons then slowly extend along cortical layers I and VI toward the caudal regions of cortex. At 16 to 32 wk, fine terminal branches from axons in layers I and VI extend radially toward the middle cortical layers and axonal density slowly increases toward normal levels although reinnervation in occipital cortex and more posterior cortical regions show the greatest delay in regeneration (Molliver *et al.*, 1989; 1990). Thus our observations are in agreement with the hypothesis that cortical serotonin axons regenerate in a rostral-caudal gradient. A rostral-caudal pattern of recovery for 5-HT uptake sites was also observed in the hippocampus. The observed temporal recovery (of 5-HT uptake sites first in rostral followed by caudal hippocampus) is similar to the temporal pattern observed for serotonin innervation of the developing hippocampus (Azmitia and Segal, 1978; Tork, 1985). In ontogeny studies in rat, the dorsal and medial portions of the hippocampus are innervated first by 5-HT axons from the median raphe via the cingulum bundle and fornix-fimbria pathways. This is later followed by innervation of the ventral hippocampus by axons from the dorsal raphe via the amygdalofugal pathway (Azmitia and Segal, 1978; Jacobs and Azmitia, 1992).

The observed rostral-caudal pattern of recovery for 5-HT uptake sites in cortex and hippocampus demonstrates regeneration of 5-HT axons varies from region to region. Furthermore, different rates of recovery of 5-HT uptake sites was also observed in ventromedial hypothalamus and laterodorsal thalamus. Full recovery of serotonin uptake sites in ventromedial hypothalamus occurred by 16 wks after MDMA

treatment, while only partial recovery was observed in laterodorsal thalamus at 52 wk. Both ventromedial hypothalamus and laterodorsal thalamus are innervated by the dorsal raphe (Azmitia and Segal, 1978; Tork, 1985) consequently recovery of serotonin uptake sites in both regions should occur by regeneration of axonal processes originating from the dorsal raphe. Axt *et al.*, (1992b) have reported serotonin reinnervation is dependent on the distance from cell bodies thus it would be anticipated that the recovery time in these two regions should be similar due to their close proximity. However, serotonergic recovery in ventromedial hypothalamus was considerably greater than in laterodorsal thalamus. One explanation is that in addition to axonal regeneration from the dorsal raphe, collateral sprouting may have occurred from nearby unaffected serotonin axons in the medial forebrain bundle in the lateral hypothalamus. Collateral sprouting from unaffected neurons has been observed in lesion studies with 5,6-DHT (Bjorklund *et al.*, 1973) and 5,7-DHT (Jacobs and Azmitia, 1992). In such studies, unaffected 5-HT axons in the fornix fimbria were observed to grow or sprout into the space left by the damaged cingulate bundle neurons within 4 to 14 days of degeneration of the affected axon. Inasmuch as the lateral hypothalmus was unaffected by MDMA, it is possible that sprouting may have occurred from unaffected axons in this region in addition to neuronal regeneration from the dorsal raphe to reinnervate the ventromedial hypothalamus.

An interesting observation in the course of these studies was that the recovery of 5-HT uptake sites in MDMA-treated brains never exceeded that in saline-treated brains. However, in the companion paper (Sabol *et al.*, 1996), 5-HT levels was reported to be greater in the hypothalamus of MDMA-treated animals than in those from saline-treated animals, indicating hyperinnervation. One possible explanation for the discrepancy is that there may be an increase in synthesis of serotonin. Another possibility is that the medial forebrain bundle (which traverses through the lateral hypothalamus) contains regenerated axons that are traveling from the dorsal and median raphe to target terminal regions and serotonin has accumulated at the ends of the regenerated axons (Wiklund *et al.*, 1978).

In contrast to our observations, Scanzello (1993) have recently reported full serotonergic recovery in parietal-temporal cortex and occipital cortex within 32 to 52 wk. However, these investigators used a lower treatment regimen (*i.e.*, 10 mg/kg × 4, i.p.; 2 hr apart) than that used in our study and it is feasible that serotonergic recovery could occur more rapidly with a smaller dose. In support, studies with p-chloroamphetamine in rat have shown that regeneration of 5-HT axons is inversely related to the magnitude of the administered dose (Axt *et al.*, 1992a).

Finally, the density of serotonin uptake sites in the dorsal raphe and median raphe was unaffected by MDMA. These observations are consistent with previous autoradiography (Battaglia *et al.*, 1991) and immunohistochemical studies (O'Hearn *et al.*, 1988) with MDMA. Furthermore, similar reports of a lack of an effect on cell body regions have been reported for MDA (Battaglia *et al.*, 1987; Mamounas *et al.*, 1991) fenfluramine (Appel *et al.*, 1989; Appel *et al.*, 1990) and p-chloroamphetamine (Mamounas *et al.*, 1991; Sanders-Bush and Steranka, 1978). In addition, a lack of effect by MDMA was also observed in lateral hypothalamus and ventral tegmental area and is consistent with previous reports (Battaglia *et al.*, 1991).

In summary, our study is consistent with previous reports describing the selective neurotoxicity and regional specificity of MDMA for serotonin neurons and their terminal fields. Evidence was presented demonstrating serotonin recovery from MDMA neurotoxicity was region specific and occurs at different rates and that recovery of 5-HT axons in cortex and hippocampus follows a rostral-caudal gradient.

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