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Age Modulates the Long-Term but not the Acute Effects of the Serotonergic Neurotoxicant 3,4-Methylenedioxymethamphetamine

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ABSTRACT

Tissue levels of serotonin (5-HT), levels of its metabolite 5-hydroxyindoleacetic acid (5-HIAA) and populations of 5-HT reuptake sites were measured in the brains of rats exposed to 3,4-methylenedioxymethamphetamine (MDMA) at selected developmental ages. MDMA exposure at postnatal day (PND) 10 did not result in altered 5-HT or 5-HIAA levels 1 week after administration in any brain region examined. However, MDMA exposure at PND 40 and PND 70 resulted in dose-dependent reductions in 5-HT and 5-HIAA levels at 1 week in all brain regions examined. Time course studies revealed that at PND 10, MDMA acutely (≤24 hr) reduced 5-HT levels and that these levels later recovered to control levels. MDMA also acutely reduced 5-HT levels at PND 40 and PND 70, but at these ages the 5-HT levels were persistently depressed (≥72 hr). Time course studies also revealed that MDMA acutely elevated dopamine levels in caudate putamen at PND 40 and PND 70, but no alterations in dopamine levels were observed at PND 10. Analysis of 5-HT reuptake site populations revealed that at PND 10 and PND 40, MDMA had little effect on reuptake site populations. At PND 70, however, MDMA reduced 5-HT reuptake site populations as early as 24 hr after administration. These experiments demonstrate not only that the biochemical effects of MDMA exposure are altered by the developmental status of the experimental animal, but also that each individual biochemical component may show differing sensitivities to alteration by MDMA at different developmental ages.

MDMA has been demonstrated to be a potent and selective neurotoxicant to the central serotonergic neurotransmitter system. Long-term reductions of neurochemical markers of serotonergic function are observed after administration of this compound to experimental animals. These include persistent reductions in levels of 5-HT and of its metabolite 5-HIAA (Ali *et al.*, 1989; Schmidt, 1987; Slikker *et al.*, 1989; Slikker *et al.*, 1988); persistent loss in the activity of the rate-limiting enzyme for 5-HT synthesis, tryptophan hydroxylase (Schmidt and Taylor, 1987; Stone *et al.*, 1989; Stone *et al.*, 1988) and long-term reductions in 5-HT reuptake site populations (Battaglia *et al.*, 1987; Schmidt *et al.*, 1987). The persistent reductions of these neurochemical indices have been shown to correlate with diminished immunohistochemical staining of serotonergic innervation in the CNS (O'Hearn *et al.*, 1988; Scallet *et al.*, 1988; Slikker *et al.*, 1988).

Both 5-HT and DA have been demonstrated to play a role in the expression of the serotonergic neurotoxicity induced by MDMA. For example, if MDMA-induced 5-HT release is blocked by the administration of the serotonin reuptake inhibitor fluoxetine, the serotonergic neurotransmitter system is spared the neurotoxic effects of MDMA (Berger *et al.*, 1992; Hekmatpanah and Peroutka, 1990; Schmidt, 1987; Schmidt *et al.*, 1987). On the other hand, pretreatment with a 5-HT_2 receptor antagonist such as ritanserin or ketanserin has been shown to attenuate long-term MDMA-induced 5-HT depletions by blocking serotonergic neurotransmission *via* these receptors (Nash, 1990; Schmidt *et al.*, 1991b; Schmidt *et al.*, 1990a; Schmidt *et al.*, 1990b).

Manipulations of the dopaminergic neurotransmitter system have also been shown to modify the ability of MDMA to produce persistent alterations in biochemical indices of serotonergic neurotransmitter system function. In general, treatments that enhance dopaminergic outflow tend to increase the neurotoxic effect of MDMA on the serotonergic neurotransmitter system, and treatments that reduce dopaminergic outflow tend to decrease neurotoxicity. In this manner, administration of L-DOPA, which increases DA concentra-

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ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; 5-HT, 5-hydroxytryptamine, serotonin; 5-HIAA, 5-hydroxyindoleacetic acid; DA, dopamine; DOPAC, dihydroxyphenylacetic acid; HVA, homovanillic acid; FRCX, frontal cortex; HPC, hippocampus; CPu, caudate putamen; HPLC, high-performance liquid chromatography; ECD, electrochemical detection; PND, postnatal day; 5,7-DHT, 5,7-dihydroxytryptamine; PCA, p-chloroamphetamine; FEN, fenfluramine.

tions in the central nervous system, concomitantly with MDMA enhances the long-term 5-HT-reducing effects of MDMA exposure (Schmidt *et al.*, 1991a,b; Schmidt *et al.*, 1990c). Conversely, DA synthesis inhibition and lesions of the substantia nigra, treatments that reduce central nervous system DA concentrations, diminish MDMA-induced serotonergic neurotoxicity (Schmidt *et al.*, 1990c; Stone *et al.*, 1988).

Both the serotonergic and the dopaminergic neurotransmitter systems undergo significant maturation during development of the mammalian central nervous system. This maturation includes the ontogeny of 5-HT and DA concentrations as well as their respective receptor and reuptake systems. In the rat, the development of 5-HT and DA concentrations is primarily a postnatal event, coinciding with the brain growth spurt (Dobbing and Sands, 1979; Herregodts *et al.*, 1990; Nomura *et al.*, 1976). Therefore, levels of these neurotransmitters are low at birth, with whole-brain levels being less than 10% of the adult values. Concentrations of 5-HT and DA increase rapidly during the first 4 postnatal weeks, being preceded by the development of the rate-limiting enzymes responsible for their synthesis: tryptophan hydroxylase and tyrosine hydroxylase (Breese and Traylor, 1972; Deguchi and Barchas, 1972; Porcher and Heller, 1972). As concentrations of 5-HT and DA increase, so do populations of their respective pre- and postsynaptic receptors and high-affinity reuptake sites (Bruinink *et al.*, 1983; Kirksey and Slotkin, 1979; Whitaker-Azmitia *et al.*, 1990).

Because 5-HT_2 receptors, 5-HT reuptake sites, and both 5-HT and DA concentrations have been demonstrated to play an important role in MDMA-induced serotonergic neurotoxicity, it would seem likely that the effects of MDMA on the serotonergic neurotransmitter system would vary with developmental status. In support of this hypothesis, a potential role for developmental age as a modulator of MDMA-induced serotonergic neurotoxicity was recognized when multiple doses of MDMA administered during early and mid-gestation in the rat had no effect on the postnatal neurochemical development of the serotonergic neurotransmitter system (St. Omer *et al.*, 1991). Similarly, p-chloroamphetamine and fenfluramine, chemical relatives of MDMA, do not produce long-term deficits in 5-HT concentrations when administered to the rat during early postnatal development (Clemens *et al.*, 1978; Clineschmidt *et al.*, 1978; Lucot *et al.*, 1981). Preliminary investigations in this laboratory have demonstrated that MDMA does not produce persistent reductions in 5-HT and 5-HIAA levels after administration to preweanling rats (Broening *et al.*, 1992). These findings demonstrate that the development of susceptibility to the long-term 5-HT-reducing effects of MDMA is primarily a postnatal phenomenon.

Inasmuch as the persistent effects of MDMA are modulated by the developmental status of the experimental animal, we hypothesized that further investigation of this phenomenon could identify a critical period for onset of sensitivity and might reveal correlates between ontological events and expression of the persistent effects of MDMA. It may then be possible to probe into the underlying mechanisms responsible for the expression of a neurotoxic insult elicited after exposure to MDMA.

In the following studies, we investigated the effects of developmental status on the acute (≤24 hr) and long-term (≥72 hr) biochemical effects induced by MDMA on the central serotonergic neurotransmitter system. The dose-response relationships of MDMA-induced neurochemical alterations were evaluated in neonatal to young adult rats. The time course of MDMA-induced serotonergic alterations was then compared and contrasted between adult and neonatal rats.

Materials and Methods

Animals and housing. Plug-positive Sprague-Dawley rats were obtained from the National Center for Toxicological Research (Jefferson, AR) breeding colony, were provided food and water *ad libitum* and were maintained in a 22 ± 1°C environment with 50% humidity. Upon parturition, offspring in each litter were counted, sexed, weighed, randomly culled to eight pups (four male and four female) and identified with foot tattoos. At PND 20, litters were weaned and animals were housed two per cage. All animals were housed in polycarbonate cages with sterilized chipped hardwood bedding and stainless steel wire covers.

Experimental design. In the first set of investigations, the long-term dose-response effects of MDMA on the central serotonergic neurotransmitter system were investigated. In the second set of investigations, the time course of MDMA-induced alterations on brain monoamine levels and 5-HT reuptake transporter populations were compared and contrasted among adult, adolescent and neonatal rats.

Dose-response investigations. (±)MDMA · HCl was obtained from the National Institute for Drug Abuse (Rockville, MD) and purity was established to be 98.2% by gas chromatography with flame ionization detection and proton NMR. MDMA was dissolved in a sterile physiological saline solution and was administered p.o. to rats on either PND 10 (10 ml/kg b.wt.), PND 40 (5 ml/kg b.wt.) or PND 70 (1 ml/kg b.wt.). At PND 10, 40 or 70, rats received a single dose of 0, 10, 20, or 40 mg/kg MDMA calculated as the free base. Rats dosed at PND 10 were immediately returned to the dam after MDMA administration. Rats dosed at PND 40 and PND 70 were housed individually before dosing and remained individually housed for the duration of the experiment. Rats were sacrificed 1 week after MDMA administration. Six litters were randomly assigned to each treatment age. Each dose level of MDMA was systematically administered to one male and one female within each litter (n = 6 rats/dose/dosing age/sex).

Time course investigations. Rats were maintained and handled as described above, and at PND 10, 40 or 70, rats received a single dose of 0 or 40 mg/kg MDMA administered p.o. Rats were sacrificed at 3, 6, 12, 24, 72, 120 and 168 hr after MDMA administration. Three litters were randomly assigned to each of the 21 levels of dosing age and sacrifice time. Each dose level of MDMA was systematically administered to two males and two females within each litter (n = 6 rats/dose/dosing age/sacrifice time/sex).

Biochemical assays. Animals were sacrificed by decapitation, and the brains were immediately removed, cooled in sterile physiological saline and dissected over ice in accordance with the method of Glowinski and Iverson (1966). Brains were dissected into FRCX, HPC and CPu, and these regions were immediately frozen on dry ice and stored at −70°C for subsequent biochemical analysis.

HPLC analysis of monoamine content. A modified procedure of Ali *et al.* (1986) was used to determine monoamine content. Briefly, tissues were weighed and diluted with 5 volumes of 0.2 N perchloric acid containing 400 nM of an internal standard, dihydroxybenzylamine. The tissue was then disrupted by ultrasonification and centrifuged at 1000 × g. Two hundred μl of the supernatant was then removed and filtered through a microfilter of pore size 0.45 μm, and 25 μl of the filtrate was injected directly onto the HPLC system. The HPLC system comprised a Waters 510 HPLC pump (Millipore/Waters Chromatography Div., Milford, MA), a Rheodyne 7125 injector (Rheodyne Inc., Cotati, CA), a Supelco Supelcosil LC-18

3 μm 4.6 × 75 mm reverse-phase analytical column (Supelco Inc., Bellefonte, PA), a BAS LC-4B amperometric detector and a flow cell (Bionalytical Systems Inc., West Lafayette, IN) consisting of a glassy carbon electrode *vs.* a Ag-AgCl reference electrode maintained at an oxidation potential of +0.70 V. The mobile phase consisted of methanol and 0.07 M monobasic potassium phosphate in a ratio of 1:9 adjusted to pH 3.0 with phosphoric acid, 1.0 mM 1-heptane sulfonic acid and 40 mg/l ethylenediaminetetraacetic acid disodium salt. Chromatograms were recorded and integrated, and the neurotransmitter concentrations were calculated from standard curves generated for each analyte of interest. Tissue concentrations were determined for DA, DOPAC, HVA, 5-HT and 5-HIAA.

5-HT reuptake site assay. A modification of the procedure of Marcusson *et al.* (1988) was used for analysis of 5-HT reuptake sites. FRCX was homogenized in 5.0 ml ice-cold 0.32 M sucrose using a motor-driven Teflon pestle and a glass mortar. The resulting homogenate was centrifuged at 40,000 × *g* for 12.5 min. The supernatant was discarded, and the resulting pellet was washed twice by resuspension in 5.0 ml ice-cold 50 mM TRIS buffer, pH 7.4 at 25°C, with 120 mM NaCl and 5 mM KCl, and was centrifuged at 40,000 × *g* for 12.5 min. After washing, the tissue pellet was resuspended in 100 volumes of TRIS buffer. Incubations were initiated with the addition of 100 μl of tissue homogenate (40–50 μg protein) to 10.0 to 1000 pmol [^{3}H]paroxetine (22.00 Ci/mmol, Dupont NEN, Boston, MA) in Tris buffer at a final volume of 2.0 ml and were allowed to reach equilibrium for 1 hr at 25°C. Nonspecific binding was determined in the presence of 100 μM 5-HT. The incubations were terminated with the addition of 4.0 ml ice-cold TRIS buffer and by filtration through glass-fiber filter paper soaked in 0.1% polyethyleneimine using a 24-well cell harvester. The filters were then washed rapidly three times with 5.0 ml ice-cold TRIS buffer, and they were equilibrated with 7.5 ml scintillation cocktail and counted on a scintillation counter at a counting efficiency of approximately 50%. Protein content was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Saturation curves were analyzed, and receptor populations and dissociation constants were determined, using the Lundon-1 saturation analysis software package (Lundon Software Inc., Chagrin Falls, OH).

Data analysis for dose-response studies. The effects of MDMA on neurochemical parameters were analyzed with a multiple fixed-factor analysis of variance model with main effects for dose, sex and dosing age, using the SAS® general linear models procedure. It should be noted that each MDMA treatment occurs an equal number of times within each litter; therefore, litters become complete blocks with respect to dose levels of MDMA. Dosing ages are unique to each litter; therefore, litters are incomplete blocks with respect to levels of dosing age and sacrifice time. Such a design is called a split-plot design (Steel and Torrie, 1980). The primary factor of interest, doses of MDMA, is assigned to all litters or blocks, because the variation within litters is expected to be less than the variation among whole units (one unit being one complete assignment of dosing ages). The appropriate error term for comparing MDMA effects against saline controls is the dosing age within, litter assignment by dose term. Linear contrasts were constructed for the comparison of MDMA effects *vs.* saline control. P values of less than .05 were accepted as statistically significant in the presence of statistical significance ($P \leq .05$) in the respective main effect or interaction.

Data analysis for time course studies. The effects of MDMA on neurochemical parameters were analyzed as described above with the addition of a main effect for sacrifice time. The appropriate error term for comparing MDMA effects against saline controls is the dosing age and sacrifice time within, litter assignment by dose term.

Results

Dose-response effects of MDMA in FRCX, HPC and CPu. MDMA administration at PND 10 did not significantly reduce 5-HT levels at 1 week after exposure in FRCX, HPC or CPu (fig. 1). However, in contrast to the lack of long-term effects after MDMA exposure at PND 10, exposure at PND 40 and 70 produced persistent dose-related decreases in 5-HT concentrations in each of the brain regions investigated. MDMA administration at PND 40 resulted in dose-related decreases in 5-HT levels in FRCX [$F(3,45) = 13.97$, $P < .001$], HPC [$F(3,45) = 21.25$, $P < .001$] and CPu [$F(3,45) = 16.60$, $P < .001$] (fig. 1). FRCX and HPC were the most severely affected regions, with 5-HT being reduced by about 15% to 45% at 1 week. 5-HT levels in CPu were the least affected in that only the highest dose of MDMA brought about a persistent reduction in 5-HT concentrations. Similar results were obtained after MDMA exposure at PND 70 in that significant dose-related decreases in 5-HT content were observed in FRCX [$F(3,45) = 88.27$, $P < .001$], HPC [$F(3,45) = 38.21$, $P < .001$] and CPu [$F(3,45) = 35.86$, $P < .001$] (fig. 1). Concentrations of 5-HT in FRCX and HPC were also the most affected at PND 70, with reductions of about 30% to 47%, whereas in CPu, 5-HT levels were reduced by 30% to 38% at 1 week.

Interestingly, the response of both the PND 40 and the PND 70 ages to the long-term 5-HT-reducing effects of 40 mg/kg was very similar in each of the brain regions examined. The major difference between the two ages was in their response to the 20-mg/kg dose: the PND 70 rats were more sensitive to long-term reductions in 5-HT at this dose than were their PND 40 counterparts (fig. 1). This difference was most striking in CPu, where 20 mg/kg MDMA did not produce reductions in 5-HT levels at 1 week in the PND 40 animals but reduced 5-HT levels by 30% in the PND 70 animals.

In general, the effects of MDMA on concentrations of the 5-HT metabolite 5-HIAA paralleled the changes observed in 5-HT at the PND 10, PND 40 and PND 70 exposure ages (data not shown). Levels of 5-HIAA in FRCX, HPC and CPu were not significantly diminished at 1 week after MDMA exposure at PND 10. As with 5-HT, 5-HIAA concentrations were most sensitive to persistent reduction in FRCX and HPC, with levels being reduced approximately 48% by 40 mg/kg MDMA at 1 week after both the PND 40 and the PND 70 exposures. However, unlike the differences between the PND 40 and the PND 70 rats in the responses of 5-HT levels to 20 mg/kg MDMA, no differences were noted between the percent reductions of 5-HIAA concentration in response to the 20 mg/kg dose at the two exposure ages. Twenty mg/kg MDMA reduced 5-HIAA concentrations by approximately 23% in FRCX but did not affect 5-HIAA levels in either HPC or CPu in both the PND 40 and the PND 70 rats.

Time-course of the effect of MDMA exposure on 5-HT levels. Administration of 40 mg/kg MDMA had a significant effect on 5-HT concentrations in FRCX at all three exposure ages (fig. 2). Reductions in 5-HT concentrations were greatest at 6 to 12 hr after MDMA administration at the PND 10, PND 40 and PND 70 exposure ages. The greatest decrease in 5-HT concentrations after PND 10 exposure was 77%, whereas after the PND 40 and PND 70 exposures, the largest reductions in 5-HT levels were about 87%. Although the PND 40 and PND 70 rats exhibited a larger percent reduction in 5-HT levels in FRCX over their PND 10 counterparts, the actual values of 5-HT concentrations at their nadir were similar at all three exposure ages, being in the range of 145 to 208 pmol per gram of tissue.

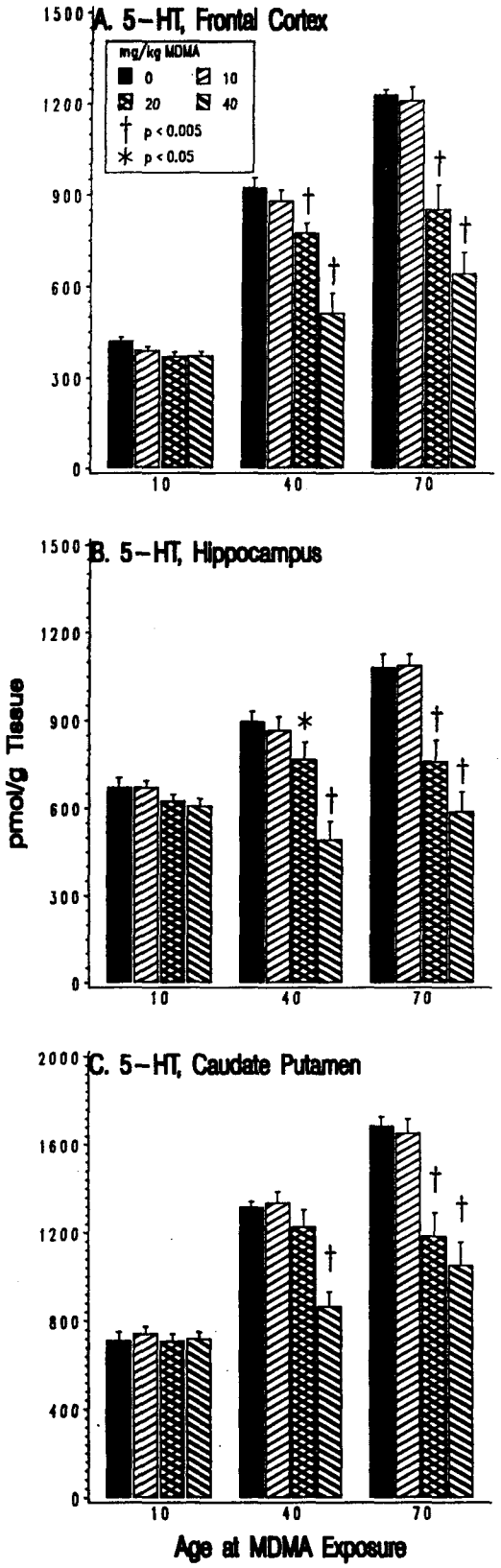

Fig. 1. Effects of MDMA on 5-HT levels in FRCX, HPC and CPu. 0, 10, 20 or 40 mg/kg MDMA was administered at PND 10, PND 40 or PND 70, and the animals were sacrificed 1 week later. Values from male and female rats have been pooled in the absence of differences in the responses of the two sexes to MDMA. Results are expressed in picomoles per gram of tissue, and each bar represents the mean of 12 rats ± S.E. * $P < .05$, † $P < .005$ *vs.* saline control.

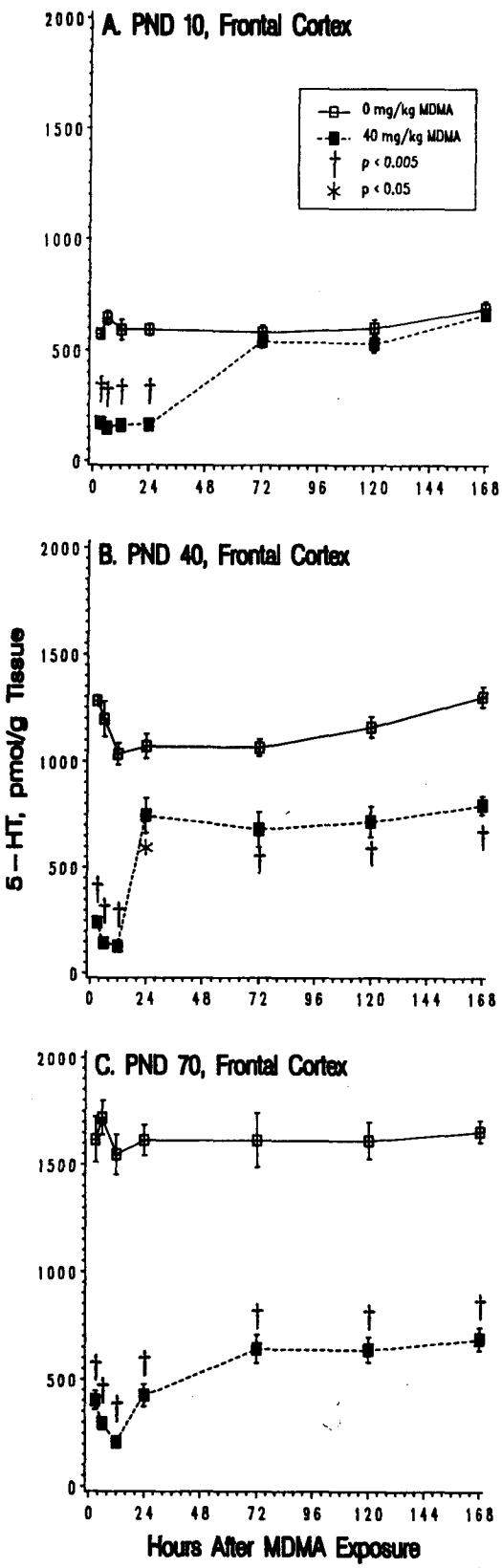

Fig. 2. Time course of MDMA-induced 5-HT reductions in FRCX. 0 or 40 mg/kg MDMA was administered at PND 10, PND 40 or PND 70, and the animals were then sacrificed at 3, 6, 12, 24, 72, 120 and 168 hr after MDMA exposure. Values from male and female rats have been pooled in the absence of differences in the responses of the two sexes to MDMA. Results are expressed in picomoles per gram of tissue and are represented as means ± S.E. * $P < .05$, † $P < .005$ *vs.* saline control.

The major differences between the three exposure ages in their response to MDMA exposure began to be manifest after the 24-hr time-point. In general, the recovery of 5-HT concentrations toward their control levels was decreased as the age at which MDMA was administered was increased. Therefore, 5-HT levels recovered to within control values after MDMA exposure at PND 10 but remained depressed 24 hr after MDMA exposure at PND 40 and PND 70, being reduced by 39% and 59%, respectively, for the two exposure ages. An interesting observation to note here is that the time course curves for 5-HT levels after MDMA exposure are virtually superimposable for all three exposure ages, whereas the time-course curves for the saline-treated controls exhibit a significant age-related increase in 5-HT concentrations (fig. 2; table 1). The time course of MDMA-induced alterations in 5-HT content was also investigated in HPC and CPu at PND 10, PND 40 and PND 70. The effects of MDMA on 5-HT levels in these regions paralleled those observed in FRCX (data not shown).

Time course of the effect of MDMA exposure on 5-HT reuptake sites in FRCX. Analysis of 5-HT reuptake sites in FRCX revealed that MDMA administration did not significantly reduce populations of the 5-HT reuptake carrier at any of the three exposure ages when 5-HT concentrations were at their nadir (fig. 3). MDMA exposure at PND 10 did not alter populations of 5-HT reuptake sites at any time-point investigated, and in contrast to the long-term 5-HT-reducing effects of MDMA in PND 40 animals, 5-HT reuptake sites were minimally affected at this age as well. MDMA administration at PND 40 altered 5-HT reuptake site populations only at 1 week after exposure; at this time, populations were reduced by about 21%. However, in contrast to the earlier exposure ages, MDMA administration at PND 70 resulted in losses in populations of the 5-HT reuptake site in FRCX as early as 24 hr after MDMA exposure. Populations of the 5-HT reuptake site declined progressively at all time-points investigated after the 24-hr time-point, so that at 1 week after MDMA administration, 5-HT reuptake sites were reduced by about 62% in the PND 70 animals. MDMA administration was not observed to alter the dissociation constant of [^{3}H]paroxetine for the 5-HT reuptake site at any exposure age or time-point investigated (data not shown).

Time course of the effect of MDMA exposure on DA in CPu. MDMA administration resulted in an acute increase in DA concentrations in CPu at both the PND 40 and the PND 70 exposure ages but not at the PND 10 exposure age (fig. 4). A general trend was noted for the acute effects of MDMA on DA concentration in that as DA levels significantly increased with developmental age, so did the ability of MDMA exposure to stimulate increases in DA concentrations in CPu (fig. 4; table 1). DA concentrations were noted to be at their highest in the rats treated with MDMA at PND 40 and PND 70 at the 3-hr time-point, at which time the levels had increased by about 17% and 23%, respectively, in CPu. Alterations in the DA metabolites DOPAC and HVA also were observed after MDMA exposure. In contrast to the alterations in DA levels, which were observed in the PND 40 and PND 70 animals only, alterations in the concentrations of DOPAC and HVA were observed at all three exposure ages. There was a general trend of MDMA to reduce acutely the levels of these DA metabolites in the PND 10, PND 40 and PND 70 animals. With a few exceptions (notably a small reduction in DA levels at 72 hr and a delayed recovery of HVA concentrations in the PND 70 animals), the DA, DOPAC and HVA levels returned to within their control values by 72 hr at all three exposure ages.

Discussion

These studies use the criteria of long-term reductions in biochemical indices of serotonergic neurotransmitter system function (population of 5-HT reuptake sites and levels of 5-HT and 5-HIAA) to investigate the neurochemical effects of MDMA on the rat CNS during three periods of development. These criteria are not meant to be all-encompassing but rather were selected so that we could investigate a specific set of events that occur after MDMA exposure. We do not content that MDMA is without adverse effect in the neonatal rat, but only that the neonatal rat is resistant to the long-term biochemical effects of MDMA on the serotonergic neurotransmitter system.

The finding that exposure of immature rats to MDMA does not result in long-term reductions in 5-HT concentrations or in populations of 5-HT reuptake sites was not unexpected, considering that investigations with compounds of similar chemical structure, pharmacological properties and toxicological effects have failed to demonstrate a prolonged effect on serotonin neurochemistry when MDMA is administered to preweanling rats. In general, the results presented here concerning neonatal exposures of MDMA agree with the results of Clemens *et al.* (1978) and Lucot *et al.* (1981) in studies on PCA administration in immature rats. These investigations demonstrated that immature rats are refractory to the long-term 5-HT-depleting effects of PCA exposure. In addition, PCA exhibited a similar time course to that of MDMA in its effects on 5-HT levels after neonatal administration: PCA also acutely reduced 5-HT levels, and these levels recovered after about 72 hr (Lucot *et al.*, 1981). Studies on neonatal

TABLE 1

The influence of age on the control values of 5-HT in FRCX and DA in CPu

The results are expressed in pmol/g tissue wet wt. and are presented as mean ± S.E. The values presented are from the 3-hr control animals used in the time-course studies.

Age (Days)	FRCX		CPu		
	5-HT	5-HIAA	DA	DOPAC	HVA
10	569 ± 19[a]	309 ± 24[b]	20999 ± 320[a]	3347 ± 94[a]	2855 ± 101[a]
40	1279 ± 25	462 ± 22	54617 ± 1679[c]	6913 ± 247[b]	3956 ± 144
70	1615 ± 109	596 ± 58	62884 ± 1692	7871 ± 325	4603 ± 211

[a] Significantly different from PND 40 value, $P < .005$.
[b] Significantly different from PND 70 value, $P < .05$.
[c] Significantly different from PND 70 value, $P < .005$.

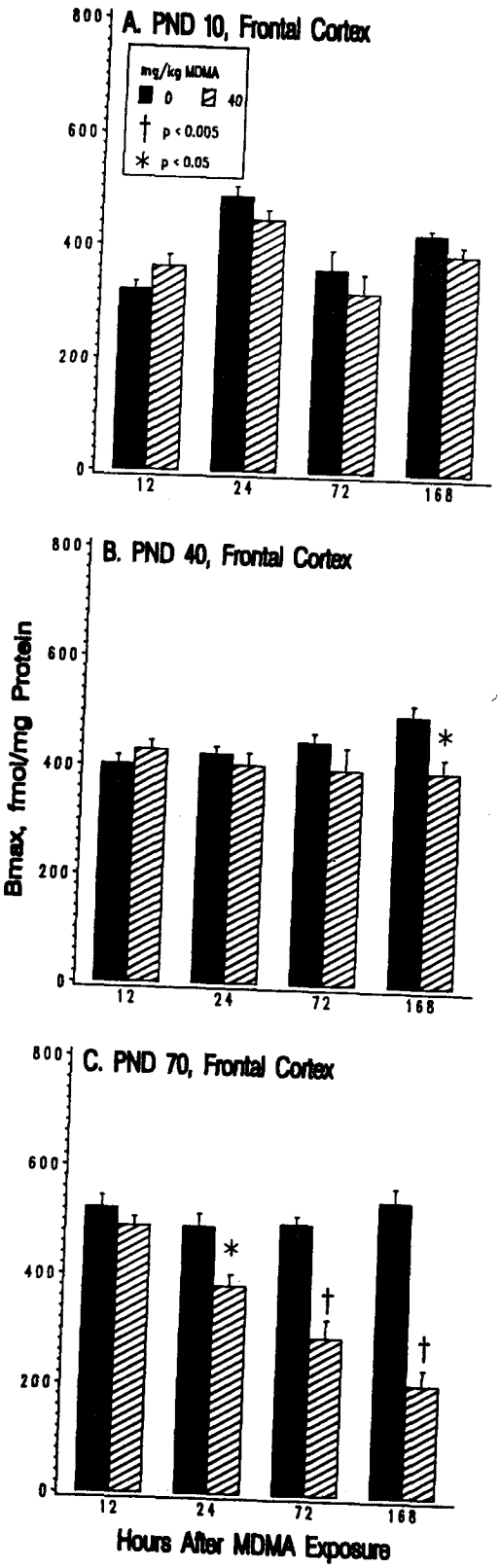

Fig. 3. Time course of alterations of 5-HT reuptake site populations after MDMA administration. 0 or 40 mg/kg MDMA was orally administered to rats at PND 10, PND 40 or PND 70, and the animals were sacrificed at times described previously. Values from male and female rats have been pooled in the absence of differences in the responses of the two sexes to MDMA. Results are expressed in femtomoles [^{3}H]paroxetine bound per milligram of protein, and each bar represents the mean of 12 rats ± S.E. * $P < .05$, † $P < .005$ *vs.* saline control.

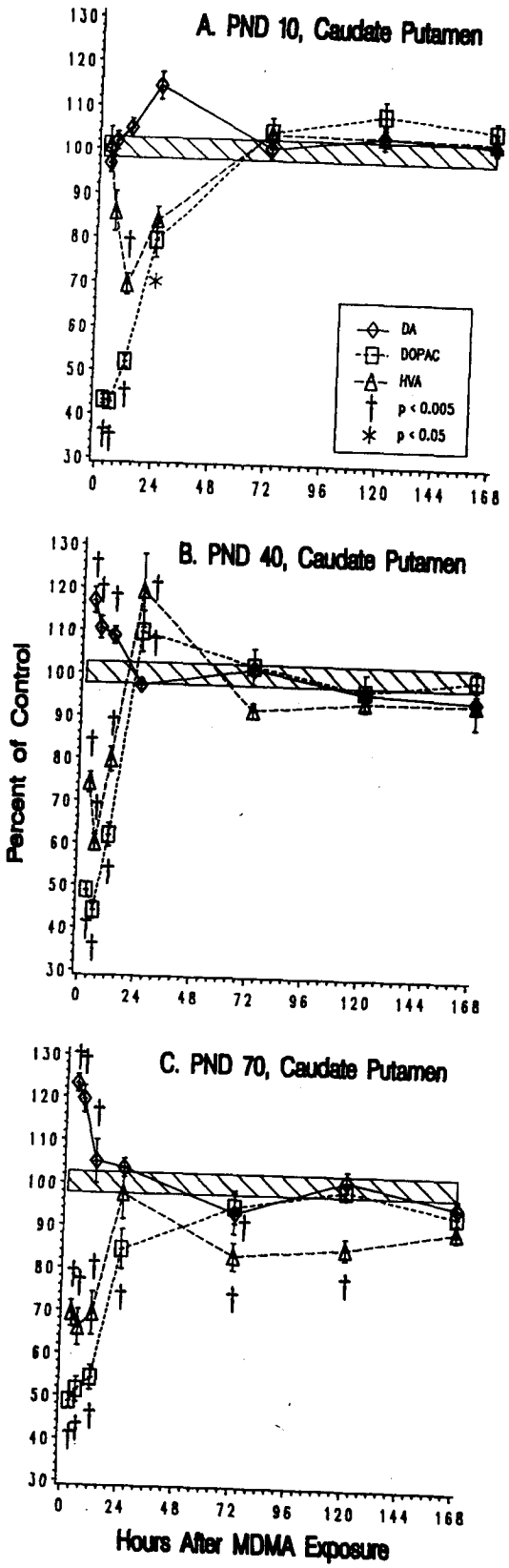

Fig. 4. Time course of MDMA-induced DA, DOPAC and HVA alterations in CPu. 0 or 40 mg/kg MDMA was administered at PND 10, PND 40 or PND 70, and the animals were then sacrificed at the times described previously. Values from male and female rats have been pooled in the absence of differences in the responses of the two sexes to MDMA. Results are represented as percent of control ± S.E. * $P < .05$, † $P < .005$ *vs.* saline control.

FEN exposure offer comparable results as well. Clineschmidt *et al.* (1978) studied the ability of FEN exposure at PND 1 to produce long-term 5-HT depletions and found that FEN also did not persistently reduce 5-HT levels in neonatal rats. Our additional finding that MDMA exposure in neonatal rats did not diminish populations of 5-HT reuptake sites demonstrates further the insensitivity of immature rats to the persistent neurochemical effects of these amphetamine derivatives.

One possible explanation for the lack of sensitivity of the neonatal rat to the long-term 5-HT-reducing effects of MDMA is that the rapid growth and development of the rat pup during the period of MDMA exposure may result in a rapid recovery of neurochemical parameters after MDMA administration. However, data from the time course studies argue against this hypothesis, because the recovery of 5-HT levels after MDMA exposure is much more rapid than the normal development of levels of 5-HT concentrations observed in the respective control animals. Furthermore, no reductions in 5-HT reuptake sites were observed to occur after MDMA exposure at PND 10. It has been inferred from the loss of 5-HT reuptake site populations that a loss of presynaptic serotonergic nerve terminals has occurred (Battaglia *et al.*, 1987). Therefore, it can be assumed that no structural damage has occurred to the serotonergic neurotransmitter system that would lend itself to persistent reductions in 5-HT levels.

As our data demonstrate, the serotonergic neurotransmitter system is not completely sensitive to the MDMA-induced loss of 5-HT concentrations and 5-HT reuptake site populations until sometime between PND 40 and PND 70. The exact age at which the serotonergic neurotransmitter system becomes fully sensitive to these MDMA-induced alterations is not known but is under investigation. However, the serotonergic neurotransmitter system is vulnerable to neurotoxic insult at very early ages, as is evidenced by the observation that administering 5,7-DHT to neonatal rats produces long-term reductions in 5-HT concentrations and [^{3}H]5-HT uptake (Breese *et al.*, 1978; Jonsson *et al.*, 1978; Towle *et al.*, 1984). This suggests that MDMA is not a direct-acting neurotoxicant, as 5,7-DHT is postulated to be (Jonsson, 1983). This assertion is further supported by the observation that MDMA does not alter immunohistochemical or neurochemical serotonergic endpoints after intracerebral administration, as does 5,7-DHT (Ali *et al.*, 1990; Molliver *et al.*, 1986). If MDMA is not a direct-acting neurotoxicant and because the long-term biochemical effects of MDMA are modulated by developmental age, then it seems likely that certain maturational events are responsible for the increase in sensitivity to MDMA that occurs during development. Although the identity of these maturational events is at present unknown, there exist several likely avenues for conjecture.

From the data presented, it is readily apparent that a significant degree of development of 5-HT levels is occurring between the different age groups. 5-HT levels show age-related increases at the PND 40 and PND 70 exposure ages. It has been proposed that efflux of neurotransmitter, which is noted to occur after the administration of amphetamine, methamphetamine, MDMA and other amphetamine derivatives, may be responsible for the induction of neurotoxicity after exposure to MDMA (Rudnick and Wall, 1992; Schmidt *et al.*, 1987). If so, then the data presented here may argue for the existence of a threshold level of 5-HT release that must be exceeded in order for persistent alterations in serotonergic endpoints to be expressed. The increased availability of 5-HT for release by MDMA may be responsible for the increase in sensitivity at each exposure age to the long-term effects of MDMA.

Several investigators have demonstrated that the efflux of dopamine induced by MDMA plays an important role in the expression of serotonergic neurotoxicity after MDMA administration (Schmidt *et al.*, 1991a,b; Schmidt *et al.*, 1990b; Stone *et al.*, 1988). The data from the time-course investigations into the effects of MDMA on concentrations of dopamine and its metabolites in CPu demonstrate not only that DA concentrations increase as the rat matures but also that the effects of MDMA on the dopaminergic neurotransmitter system are altered with developmental age. Although acute reductions in DOPAC and HVA levels were a consistent finding at all three exposure ages, MDMA-induced increases in DA levels were not noted until PND 40, an age at which 5-HT concentrations were persistently diminished by MDMA. The 5-HT$_2$ receptor has been shown to play a role in the MDMA-induced increases in DA levels in that the release of endogenous 5-HT by MDMA may enhance tissue DA levels *via* 5-HT$_2$ receptor stimulation (Nash, 1990; Nash *et al.*, 1990; Yamamoto and Spanos, 1988). Blockade of 5-HT$_2$ receptors by 5-HT$_2$ antagonists such as ketanserin and ritanserin have been demonstrated to suppress the MDMA-induced increase in DA concentrations and subsequently to diminish the long-term biochemical effects of MDMA exposure (Nash, 1990; Nash *et al.*, 1990; Schmidt *et al.*, 1991b; Schmidt *et al.*, 1990b). 5-HT$_2$ receptor populations do not reach adult levels until approximately PND 28 in the rat (Bruinink *et al.*, 1983). The lack of an effect of MDMA exposure on DA levels in CPu at PND 10 may indicate that 5-HT$_2$ receptors are not fully functional in the neonatal rat brain, that the neonatal dopaminergic neurotransmitter system is insensitive to acute effects of MDMA or a combination of both.

Adult rats demonstrate a hyperthermic response to MDMA exposure at room temperature, but at cooler ambient temperatures they exhibit a hypothermic response (Gordon *et al.*, 1991; Schmidt *et al.*, 1990b). Similar results have been demonstrated with methamphetamine (Bowyer *et al.*, 1994; Bowyer *et al.*, 1992). Interference with this hyperthermic response has been shown to diminish the long-term neurochemical effects of both MDMA and methamphetamine (Bowyer *et al.*, 1994; Bowyer *et al.*, 1992; Schmidt *et al.*, 1990b). Winslow and Insel (1990) have observed that neonatal rats administered MDMA do not exhibit a rise in core temperature. In agreement with this, we have observed that exposure of PND 10 rats to MDMA does not elicit a hyperthermic response but that exposure of PND 40 and PND 70 rats to MDMA does (unpublished observations). It may be that the lack of a hyperthermic response to MDMA administration at PND 10 contributes to the lack of susceptibility of the neonatal rat to the persistent effects of MDMA.

An interesting observation from the time course studies is that the administration of MDMA to PND 40 rats resulted in persistent reductions in 5-HT levels but had no effect on populations of 5-HT reuptake sites until 1 week after exposure. This was in contrast to MDMA administration to PND 70 rats, where reductions in 5-HT reuptake sites were observed as early as 24 hr after exposure and tended to reflect

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the losses in 5-HT concentrations at 1 week. Pu and Vorhees (1993) have recently observed a similar phenomenon in that developmental age dissociates reductions in tyrosine hydroxylase immunoreactivity from increases in glial fibrillary acid protein immunoreactivity after the administration of methamphetamine. If reductions in 5-HT reuptake site populations indicate that a loss of presynaptic serotonergic nerve terminals has occurred, then these data suggest that reductions in 5-HT concentrations can occur independently from serotonergic nerve terminal ablation. Therefore, although reductions in 5-HT concentrations and diminished populations of 5-HT reuptake sites coexist in adult rats after MDMA exposure, both effects may not be induced by the same mechanism.

In summary, the persistent effects of MDMA administration on neurochemical indices of serotonergic function in the rat are modulated by developmental status. In addition, several of the endpoints used to describe how developmental status alters the effects of MDMA exposure differed in their sensitivity to MDMA at the different ages investigated. It is our hope that adding developmental age as a modulator of neurotoxicity to the repertoire of pharmacological manipulations already available to investigate the persistent effects of MDMA exposure on the serotonergic neurotransmitter system will yield greater insight into the mechanisms whereby MDMA and its congeners produce their neurotoxic effects.

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