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Serotonergic Modulation of Rat Pup Ultrasonic Vocal Development: Studies with 3,4-Methylenedioxymethamphetamine

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ABSTRACT

3,4-Methylenedioxymethamphetamine (MDMA) has previously been shown to destroy serotonin terminals in the rat brain. Despite profound and prolonged loss of serotonin innervation, long-term behavioral effects of MDMA have not previously been reported. In this study, we monitored the short- and long-term effects of MDMA administration on the ultrasonic isolation call of the rat pup. At 30 to 60 min after a single dose of MDMA (0.5–10.0 mg/kg), isolation calls decreased as much as 90%, with a rebound increase in calling noted 10 to 25 h following administration of the highest dose. Repeated administration of 10 mg/kg MDMA (once or twice daily on postnatal days 1–4) resulted in a lasting, dose-dependent decrease in ultrasonic vocalization monitored on days 6, 9, 12 and 15. Concurrent measures of locomotor behavior, geotaxis and weight gain were not altered subsequent to repeated MDMA treatment. Both serotonin content and serotonin terminals (assessed by [^{3}H]paroxetine binding) in cortex were reduced by repeated MDMA treatment, whereas concentrations of catecholamines and their metabolites were unaltered. Repeated prenatal MDMA exposure did not affect postnatal rates of calling or the biochemical markers of serotonin in cortex. Pups lesioned with MDMA postnatally showed not only long-term behavioral and biochemical changes but also altered responsiveness to the serotonin $_{1B}$ agonist 1-[3-(trifluromethyl)phenyl]piperazine. Taken together, these studies indicate that serotonergic lesions in a sensitive phase of development can have long-term selective effects on the rat pup ultrasonic isolation call, a behavior critical for mother-infant affiliation.

The methamphetamine derivative, MDMA (also known as "Ecstasy"), has recently been shown to be a potent and selective serotonergic neurotoxin. In studies of primates (Ricuarte *et al.*, 1988a,b; Insel *et al.*, 1989) as well as rodents (Commins *et al.*, 1987; Stone *et al.*, 1986; Battaglia *et al.*, 1987a,b; 1988), systemic administration of MDMA for 4 days has been associated with a profound and enduring decrease in serotonin content and serotonin terminals throughout the forebrain. A curious aspect of this toxicity has been the apparent difficulty in demonstrating similar long-lasting changes in behavior after MDMA administration (Peroutka *et al.*, 1988; Nencini *et al.*, 1988; Spanos and Yamamoto, 1989). Central nervous system serotonin has been implicated in the mediation of sleep, appetite, anxiety and aggression (reviewed in Soubrié, 1986); however, animals with up to 90% depletion of telencephalic serotonin following MDMA treatment show no apparent deficits in these behavioral categories. In the present study, we demonstrate that MDMA administered to neonatal rats has long-term effects on a relatively unknown, but ethologically significant behavior: the ultrasonic isolation call.

The isolation call is one of the earliest and most important social behaviors expressed by virtually all mammalian species as part of the protest response to maternal separation (Panksepp, 1985). In rats and other rodent species, this call is an ultrasonic vocalization in the 30 to 70 kHz range (Insel *et al.*, 1988). Although isolation calling is present only within the first 15 days of postnatal life, during this period it is a potent stimulus for maternal activation, approach and retrieval (Smotherman *et al.*, 1974; Insel *et al.*, 1988). This call is exquisitely sensitive to ambient temperature and olfactory cues (Okon, 1971; Noirot, 1972; Hofer and Shair, 1987) and is also responsive to pharmacological treatment. Benzodiazepine and opioid agonists selectively reduce calling, whereas purported anxiogenic drugs such as pentylenetetrazol increase calling (Gardner, 1985; Insel *et al.*, 1986; Kehoe and Blass, 1986). Evidence of monoaminergic involvement in the modulation of calling has also been demonstrated. For example, the *alpha*$_2$ adrenergic receptor agonist clonidine, and the norepinephrine

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ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; TFMPP, 1-[3-(trifluromethyl)phenyl]piperazine; 8-OH-DPAT, (±)-8-hydroxy-2-(di-n-propylamino)tetralin; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; 5HT, serotonin; 5HIAA, 5-hydroxyindoleacetic acid; 5,7-DHT, 5,7-dihydroxytryptamine.

re-uptake inhibitor desmethylimipramine (Hård *et al.*, 1988; Winslow and Insel, 1990) produce dose-related increases in the rate of calling. A potential role for serotonin in the mediation of calling has also been indicated by the rate decreasing effects of serotonergic re-uptake inhibitors and $5HT_{1A}$ agonist drugs (Hård and Engel, 1988; Mos and Olivier, 1989a,b; Winslow and Insel, 1990).

In the following studies, we further investigated the role of serotonin in the infant rat pup's response to mother-infant separations. We studied the short- and long-term effects of MDMA on the vocal, motor and thermoregulatory behavior of infant rats. In addition, we assessed biochemical measures of monoaminergic depletion following administration of neurotoxic doses of MDMA. Finally, we describe alterations in the behavioral sensitivity to selective 5HT receptor ligands following MDMA lesions.

Materials and Methods

Animals and housing. Offspring of Sprague-Dawley breeders (Taconic Farms, Taconic NY) were housed with both parents in polycarbonate cages (55 × 31 × 21 cm) until 21 days old. The vivarium temperature and lighting were constant at 24°C and 14 10-hr light-dark cycles. At 30 min before testing, litters were removed as a group from their home cages and placed in new cages with a small amount of home-cage bedding. Cages were transported to a lighted testing room adjacent to the vivarium, with an ambient temperature maintained at 24 ± 1°C. Unless otherwise specified, litters were not culled prior to testing.

Behavioral testing. Beginning 30 min after removal from their home-cage, each pup was placed in a polycarbonate recording chamber (46 × 29 cm) with a 5 × 5-cm grid drawn on the floor. A microphone (Model 4385; Bruel and Kjaer, Copenhagen) with a parabolic reflector was suspended 10 cm over the cage floor to record pup ultrasonic vocalizations. Ultrasonic calls were analyzed by a digital sound spectrum analysis system providing on-line the number of calls in each 2-min session (Burkholder *et al.*, 1982; Insel *et al.*, 1986). The number of grid cells entered by the pup was also collected during this period by visual scoring. At the end of a 2-min recording period, each pup was weighed and rectal temperature sampled (probe: YSI-K74367). We also measured the latency to turn around when placed head-down on an inclined plane (geotaxic response). Pups placed head down on an inclined plane will turn against the slope, and the latency declines with age. This behavior provides an independent measure of motor development and impairment (Mos and Olivier, 1989a, b). Pups then received injections of vehicle or drug s.c. at the nape of the neck, and were returned to their littermates. Pups were retested at various time intervals after injection to assess drug effects and were again returned to littermates.

Experimental Design. In the first group of experiments, we assessed the acute effects of several doses of MDMA on infant rat behavior. In a second group of experiments, we studied the long-term effects following repeated administration of a large dose of MDMA on brain monoamine levels, neurotoxicity and behavior of infant rats.

Acute effects. (±) MDMA was dissolved in physiological saline and administered s.c. (1 ml/100 g, b.wt.) prior to testing. Treatments were administered in a systematically varied order across four litters (9–12 pups/litter, 9–11 days old, approximately 20 g b.wt.), and each pup was used only once. Two studies of acute effects were conducted: 1) the dose effect of MDMA (0.5–10.0 mg/kg, s.c.) on infant rat behavior was measured 60 min after treatment (n = 8/dose), and 2) the time course of a selected dose of MDMA (10 mg/kg, s.c.) was measured during repeated 2-min tests over a 48-hr period. Testing began 30 min after injection and used a within subjects design (n = 6). In both cases, littermates that received saline injections served as controls.

Long-term effects. Litters were culled to nine pups and housed with both parents in polycarbonate cages (55 × 31 × 21 cm) until at least 21 days old. Beginning six to eight hr after birth, pups were individually removed from their home cage, weighed, and given injections (0.1 ml/10 g b.wt.) s.c. at the nape of the neck, and returned to their home cage. Pups received six additional injections at approximately 12-hr intervals for a total of seven injections between postnatal days 1 through 4. Three treatment regimens were studied in separate animals: 1) 10 mg/kg MDMA at 12-hr intervals (high cumulative dose, 70 mg/kg; n = 17); 2) 10 mg/kg MDMA alternated with saline at 12-hr intervals (low cumulative dose, 40 mg/kg; n = 9); 3) saline twice daily (controls, n = 26). The behavioral effects of repeated MDMA injections were measured at 2, 5, 8 and 11 days after the last injection (corresponding to postnatal ages 6, 9, 12 and 15).

To characterize further the consequences of MDMA administration on development, we administered 5HT agonist drugs to nine 12-day-old pups following repeated administration of MDMA or saline. TFMPP (0.03–0.3 mg/kg, s.c.) was used for $5HT_{1B}$, 8-OH-DPAT (0.3–3.0 mg/kg, s.c.) was used for $5HT_{1A}$ and DOI (0.3 mg/kg, s.c.) was used for $5HT_2$ receptors. All drugs were prepared fresh in 0.9% saline in concentrations designed to deliver 1.0 ml/100 g body weight. Using the behavioral protocol described above, pups were tested immediately before agonist administration, given injections of doses of TFMPP, 8-OH-DPAT, DOI or saline and returned to their littermates. Pups were retested 60 min later to evaluate drug effects. Each pup received two doses of drug at 4-day intervals. The order of dose administration was systematically varied across five litters.

MDMA was provided by Errol B. De Souza (NIDA, Baltimore, MD). DOI, 8-OH-DPAT and TFMPP were purchased from Research Biochemicals Inc., Natick, MA.

Prenatal administration. The effects of repeated *prenatal* administration of MDMA were also assessed. Timed pregnant females were obtained in our breeding colony by daily inspection of vaginal aspirates. The day sperm was noted in the aspirate was designated day 1 of gestation. Beginning at 4 P.M. on gestation day 13, individually housed dams were removed from their home-cage, weighed and given i.p. injections of either saline or 10 mg/kg MDMA (0.1 ml/100 g b.wt.). Following drug or saline administration, dams were returned to their home cages. Dams received six additional injections at approximately 12-hr intervals using the same protocol. The number of offspring from drug-treated dams was recorded at parturition. Within 24 hr of parturition, all litters were culled to a maximum of nine pups, cross-fostered to untreated, lactating females and housed as described above.

Biochemical assays. On postnatal day 21, pups from each study of long-term MDMA effects were euthanized by decapitation, and brains were immediately frozen on dry ice and stored at −70°C for subsequent analysis of concentrations of brain monoamine and receptor levels. We dissected the following areas from frozen coronal slabs: neocortex, hippocampus, striatum and hypothalamus. What is herein referred to as neocortex is actually a strip through the entire thickness of the cortex, superior to the caudate nucleus and around the convexity, including parts of the cingulate, frontal and parietal cortex. Striatum refers to caudate-putamen. To ensure that biochemical measures assayed on postnatal day 21 reflected changes relevant to the interval during which behavioral observations were collected (postnatal days 6–15), we examined biochemical changes in a subgroup of 10-day-old pups (n = 6) in both the pre- and postnatal treatment studies of long-term MDMA effects. We also examined the persistence of MDMA effects on brain 5HT in a group of 42-day-old rats (n = 12).

Assay of monoamines in cortex. Brain concentrations of 5HT, 5HIAA, 3,4-dihydroxyphenylacetic acid, dopamine, homovanillic acid and norepinephrine were measured by high-performance liquid chromatography with electrochemical detection (Mefford, 1981). Dissected regions of neocortex (50–100 mg) were sonicated in 0.5 to 1.0 ml of 0.1 N perchloric acid (4°C), then centrifuged at 12,000 × g for 10 min. Aliquots (20 μl) of the supernatants were injected into a 15-cm, 3 μ, C_{18} column with a mobile phase consisting of 0.2 M NaH_2PO_4, 160 mg/l sodium octal sulfate, 120 μl/l triethylamine, 50 mg/l EDTA, 2.5% v/v acetone. pH was titrated to 3.5 with phosphoric acid.

Assay of 5HT uptake sites. Changes in monoamine content are

indicative but not demonstrative of neurotoxicity. As [^{3}H]paroxetine binds selectively to the 5HT re-uptake site, which is localized to 5HT terminals, decreases in the amount of binding may provide an index of loss of terminals (Battaglia *et al.*, 1987b). Cortical tissue adjacent to the regions used for high-performance liquid chromatography assay of monoamine content, and regions dissected from the striatum, hippocampus and hypothalamus, were placed in 50 vol of ice-cold assay buffer [50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl (pH 7.4 at 22°C)] and homogenized using a Brinkman polytron (setting 6, 20 sec). The homogenate was centrifuged at 48,000 × *g* for 10 min with a subsequent resuspension in buffer and recentrifugation. The final pellet was resuspended in the same buffer to a concentration of 15 mg wet weight/ml. [^{3}H]Paroxetine (26.5 Ci/mmol; New England Nuclear, Boston, MA) binding was carried out with minor modifications of the original protocol (Habert *et al.*, 1985). To obtain an estimate of the maximal density of [^{3}H]paroxetine-labeled serotonin uptake sites, a saturating concentration (0.25 nM) of [^{3}H]paroxetine was incubated with 1.5 mg of tissue in the presence or absence of 1 μM citalopram MDMA and citalopram (provided by Errol B. De Souza, NIDA, Baltimore, MD) in 5 ml of the assay buffer for 2 hr at 22°C. The incubation contents were filtered rapidly over 0.01% polyethyleneimine presoaked Whatman GF/C filters and washed three times with 5 ml of ice-cold buffer. Filters were then equilibrated with 10 ml of scintillation fluid and counted in a scintillation counter.

Data analysis. Drug effects on frequency of isolation calling, cell crossing, temperature and latency to geotaxic reflex were analyzed with multiple fixed-factor analyses of variance with main effects for dose and age. Dunnett's *t* tests were performed for comparison of dose effects against saline control within an age group (Winer, 1971). Changes in brain density of paroxetine binding sites, and levels of 5HT, 5HIAA, 3,4-dihydroxyphenylacetic acid, dopamine, norepinephrine and homovanillic acid were also analyzed with fixed-factor analyses of variance with main effects for dose. Dunnett's *t* tests were performed for comparison of dose effects against saline control. For two-tailed distributions, $P < .05$ was accepted as statistically significant.

Results

Acute effects of MDMA administration. MDMA (0.5–10 mg/kg, s.c.) administered to 10-day-old pups produced a profound reduction in the rate of ultrasonic calling during a 2-min separation, 60 min after injection [$F(3,28) = 29.38$, $P < .05$] (fig. 1*A*). Reduced calling was measured at all doses and was associated with a dose-related increase in the frequency of grid cell crossing [$F(3,28) = 2.99$, $P < .05$] (fig. 1*B*) and reduced latencies to geotaxic response [$F(3,28) = 4.78$, $P < .05$] (fig. 1*C*). Rectal temperature was unaffected by MDMA (saline, 34.02 ± 0.25°C; 10.0 mg/kg MDMA; 34.2 ± 0.25°C).

The time course of MDMA's effects on vocalization in 10-day-old pups measured within subjects showed a biphasic pattern in repeated 2-min separations over a 48-hr period (fig. 2); 10 mg/kg MDMA significantly reduced the frequency of ultrasonic vocalization at 30 min ($t_D = 8.62$, $P < .05$) and 3 h ($t_D = 7.68$, $P < .05$) after injection, but was associated with a significant *increase* in calling at 10 ($t_D = 3.29$, $P < .05$) and 24 hr ($t_D = 2.50$, $P < .05$). The rate of calling returned to pretreatment levels by 48 hr after injection. Similar acute effects were measured in younger pups given injections of 10 mg/kg as part of the repeated administration protocol (data not shown).

Effects of repeated administration. Repeated administration of 10 mg/kg MDMA to neonatal pups produced a dose-related, long-lasting reduction in the rate of isolation calling by pups measured up to 11 days after the last injection [$F(2,32) = 18.37$, $P < .05$] (fig. 3). Saline-treated pups showed a char-

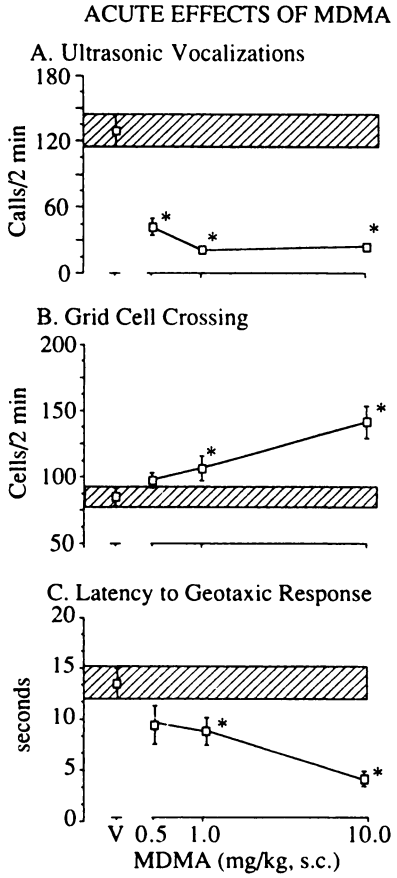

Fig. 1. Effects of acute systemic administration of MDMA (0.5–10.0 mg/kg, s.c.) measured during 2 min social isolation beginning 60 minutes after injection. Each pup was used only once, and dose assignment ($n = 9$ per dose) was systematically varied across four litters. The figures portray drug effects on the number of ultrasonic calls (top), grid cell crossings (middle), and the latency to show a geotaxic response on an inclined plane (bottom). ■, mean ± 1 S.E.M. for the saline control group. Vertical lines at each data point represent ± 1 S.E.M. * Significant Dunnett's *t* comparisons of drug dose with saline ($P < .05$), in the presence of significant overall treatment effects detected by analysis of variance.

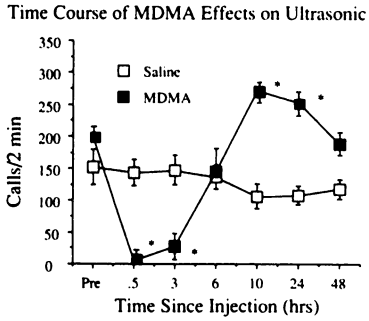

Fig. 2. Time-course of the effects of a single systemic injection of MDMA (10 mg/kg, s.c.; ■) or saline (□) on the number of ultrasonic calls during 2 min social isolation. Repeated recordings were collected from saline ($n = 6$) or MDMA ($n = 6$) treated pups immediately before injection, then 30 min, 3, 6, 10, 24 and 48 hr after injection. Pups were returned to their mother and littermates between recordings. Vertical lines at each data point represent ± 1 S.E.M. * Significant Dunnett's *t* comparisons ($P < .05$) of the rate of calling at each time-point to the preinjection rate, in the presence of a significant drug by time interaction effect detected by analysis of variance.

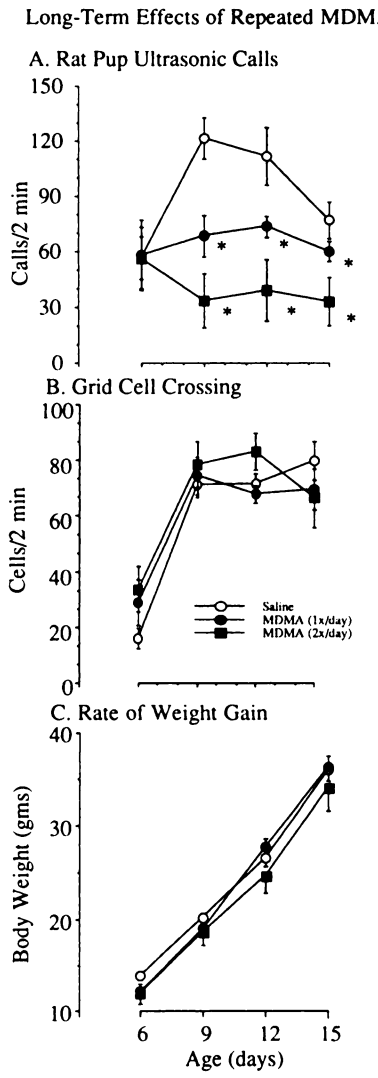

Fig. 3. Long-term effects of repeated systemic administrations of MDMA on the number of ultrasonic calls (top), grid cell crossings (middle) measured during 2 min social isolation and the rate of weight gain (bottom). Beginning within 12 hr after birth, pups were injected with saline (n = 26; ○) or MDMA at 12-hr intervals up to seven injections. MDMA-treated pups were administered 10 mg/kg MDMA either every injection (*i.e.*, 2×/day; n = 17; ■) or alternated with a saline injection (*i.e.*, 1×/day; n = 9; ●). Testing was scheduled 2, 5, 8 and 11 days after the last injection (*i.e.*, postnatal days 6, 9, 12 and 15). Vertical lines at each data point represent ± 1 S.E.M. * Significant Dunnett's t comparisons of dose of MDMA with saline control at each age ($P < .05$), in the presence of significant age by treatment effect detected by analysis of variance.

acteristic age-related increase in the rate of vocalizations between days 6 and 12 postnatal, with a subsequent decline on day 15. A similar developmental pattern for ultrasonic vocalizations has been previously reported (Insel *et al.*, 1988). A significant interaction between age and dose was detected [$F(3,58) = 3.87$, $P < .05$]. The normal developmental pattern of vocal behavior seen in the saline-treated pups was absent in the MDMA-treated pups, which showed low rates of calling from postnatal days 9 to 15 (figs. 3 and 4). These effects appeared to be relatively specific since motor activity and weight gain were not significantly affected. In addition, no significant treatment effects were noted for geotaxis or rectal temperature across the 6 to 15-day age interval.

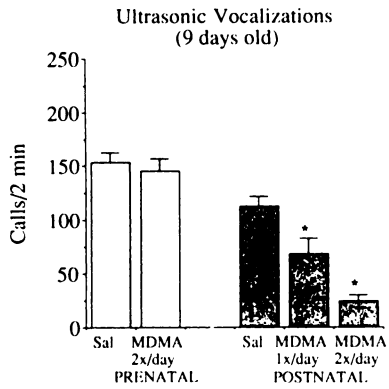

Fig. 4. Long-term effects of the repeated prenatal administration of MDMA (10 mg/kg, 2×/day, gestation days 13–16; saline, n = 18; MDMA, n = 11) on ultrasonic vocal behavior are presented with the behavior of postnatally treated pups described in figure 3 on postnatal day 9. Vertical lines at each column represent 1 S.E.M. * Significant Dunnett's t comparisons of dose of MDMA with saline control ($P < .05$), in the presence of significant overall treatment effects detected by analysis of variance.

Long-term behavioral changes following repeated administration of MDMA were associated with significant changes in biochemical measures of brain monoamines. In neocortex (fig. 5), MDMA produced a significant dose-related decrease in the concentration of 5HT [$F(2,37) = 21.81$, $P < .05$], 5HIAA [$F(2,37) = 13.09$, $P < .05$] and the number of [^{3}H]paroxetine binding sites [$F(2,37) = 16.50$, $P < .05$]. Depletion of 5HT in neocortex at the high dose of MDMA did not depend on age of sacrifice (10, 21 or 42 days old). Catecholamine and metabolite concentrations in the neocortex of 10- and 21-day-old pups were unaffected by MDMA (table 1). Analysis of serotonergic depletion in other brain areas revealed evidence of regional specificity. Twice-daily administration of 10 mg/kg MDMA reduced [^{3}H]paroxetine binding in the neocortex and striatum of 21-day-old pups by 51.4 ± 6.7% and 43.4 ± 7.5%, respectively. Binding in the hippocampus was reduced by 20.8 ± 8.3% and in the hypothalamus by 22.3 ± 4.9% (fig. 6).

A second study of repeated administration of MDMA scheduled injections during the peak period of neuronal differentiation from embryonic day 13 to 16. Repeated prenatal administration of MDMA did not significantly affect the vocal or motor activity (fig. 4) of nine-day-old pups, but did reduce both body weight (mean weight difference, 2.54 g, $t = 2.77$; $P < .05$) and core temperature (mean temperature difference, 1.03°C, $t = 3.02$, $P < .05$) compared with saline-treated controls. No significant differences in cortical markers of 5HT or CAs were detected in these animals when measured on postnatal day 10 or 21 (fig. 5; table 1).

Response to subtype selective 5HT receptor agonists. To characterize further the effects of repeated postnatal treatment with MDMA (10 mg/kg), we administered various 5HT receptor agonists to pups beginning 4 days after the last injection of MDMA or saline. The $5HT_{1A}$ agonist 8-OH-DPAT produced a dose-related decrease in the rate of calling in both saline control (injected repeatedly with saline on days 1–4) and MDMA-treated pups [$F(3,74) = 14.30$; $P < .05$] (fig. 7). 8-OH-DPAT also produced dose-related decreases in the frequency of cell crossing [$F(3,74) = 6.52$; $P < .05$] and core temperature [$F(3,74) = 12.62$; $P < .05$] independent of neonatal treatment.

Administration of the $5HT_{1B}$ agonist TFMPP produced a significant, biphasic dose-related change in the rate of ultrasonic calling [$F(3,74) = 13.23$; $P < .05$] with low doses increasing

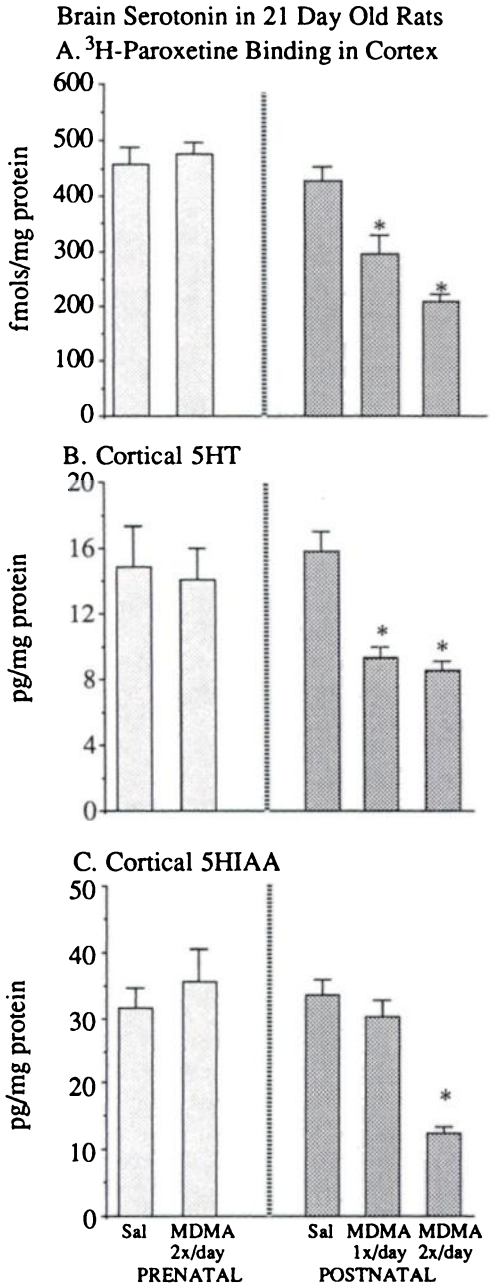

Fig. 5. Effects of repeated systemic administrations of MDMA on the average cortical density of [^{3}H]paroxetine uptake sites (top), content of 5HT (middle) and 5HIAA (bottom). Postnatally and prenatally treated pups with behavioral effects portrayed in figure 3 were sacrificed on postnatal day 21. An estimate of the maximal density of [^{3}H]paroxetine-labeled serotonin uptake sites was determined in the presence of a saturing concentration (0.25 nM) of [^{3}H]paroxetine. Brain concentrations of 5HT and 5HIAA were measured by high-performance liquid chromatography with electrochemical detection. Vertical lines at each column represent 1 S.E.M. * Significant Dunnett's *t* comparisons of dose of MDMA with saline control within each treatment group ($P < .05$), in the presence of significant overall treatment effects detected by analysis of variance.

calling and higher doses not affecting or reducing the rate of calling. A trend in the interaction between TFMPP and neonatal treatment [$F(1,75) = 2.34$; $P = .08$] appeared to be accounted for by a reduced sensitivity to the effects of TFMPP in MDMA-treated pups. In MDMA-treated pups, TFMPP did not significantly increase the number of ultrasonic vocalizations. TFMPP also produced a dose-related decrease in core temperature independent of neonatal treatment [$F(3,74) = 7.90$; $P < .05$], but did not affect motor activity or geotaxis at the doses tested.

Administration of the $5HT_2$ agonist DOI (0.3 mg/kg) significantly reduced the rate of calling [$F(1,35) = 11.90$; $P < .05$] and motor activity [$F(1,35) = 4.62$; $P < .05$] independent of neonatal treatment. Core body temperature and latency to geotaxic response were unaffected at this dose.

Discussion

The major findings of these studies are as follows. First, MDMA given to 10-day-old rat pups in a dose range from 0.5 to 10.0 mg/kg reduced the rate of ultrasonic vocalization up to 3 hr after injection. A subsequent increase in calling was measured between 10 and 24 hr after injection of the highest dose. Following repeated administration of MDMA to newborn pups, there was a dose-dependent, persistent decrease in ultrasonic vocalization without concurrent changes in several other behavioral and developmental parameters. Repeated postnatal administration of MDMA also produced a selective reduction of neocortical serotonin content associated with an apparent loss of serotonin terminals ([^{3}H]paroxetine binding). A significant, selective decrease in serotonin was measured as early as 6 days after the last injection (postnatal day 10) and was still evident at 38 days after treatment (postnatal day 42). Prenatal MDMA treatment (gestational days 13 to 16) did not alter either ultrasonic calling or cortical serotonin content at any age. Finally, postnatal MDMA not only appears to lesion 5HT pathways but also may alter responsiveness to the serotonin receptor ligand TFMPP ($5HT_{1B}$). These data provide strong evidence that brain serotonin has an important role in the modulation of infant rat ultrasonic calling during maternal separation.

The acute biphasic effects on ultrasonic calling produced by MDMA may be related to the two phases of MDMA actions on cortical 5HT concentrations previously reported (Schmidt, 1987). In adult rats, there is a profound decrease in 5HT neuronal content up to 6 hr after administration of 10 mg/kg MDMA, followed by recovery to basal levels by 12 hr with a subsequent gradual, long-term decline in 5HT, 5HIAA and [^{3}H] 5HT uptake sites (Schmidt, 1987). The early phase of MDMA activity is reversible and appears to involve stimulation of 5HT release (Schmidt *et al.*, 1986). It is important to note that the acute effects of MDMA also include stimulation of dopamine release up to 3 hr after administration, particularly in nucleus accumbens and caudate (Yamamato and Spanos, 1988; Schmidt, 1987). The initial decrease in ultrasonic calling rate reported here might thus result from either 5HT or dopamine release. Earlier studies with monoamine uptake inhibitors reported decreases in calling following administration of the 5HT re-uptake inhibitor clomipramine and increases following the dopamine re-uptake inhibitor mazindol (Winslow and Insel, 1990; Mos and Olivier, 1989a, b). Thus the effects of MDMA on ultrasonic calling up to 6 hr after injection appear to be most consistent with effects on serotonergic and not dopamine release. As further evidence of a serotonergic role in the inhibition of calling, $5HT_{1A}$ and $5HT_2$ agonists have been shown to decrease calling, whereas dopaminergic *antagonists* decrease calling (Cagiano *et al.*, 1986). Of course other relevant neuro-

TABLE 1

Effects of repeated prenatal and neonatal administration of MDMA (10 mg/kg, s.c.) on norepinephrine, dopamine and 5HT markers in the infant rat brain

Cortical concentrations of monoamines and metabolites following repeated MDMA (10 mg/kg) were measured using high-performance liquid chromatography. Pups were sacrificed on postnatal day 10 or 21. Because no difference by age was detected, values represent means ± S.E.M., pg/mg protein collapsed across age.

	NE*	DA	5HT	HVA	DOPAC	5HIAA
Prenatal						
Saline	1.07 ± 0.16	5.7 ± 2.6	14.8 ± 5.1	10.1 ± 1.5	9.9 ± 3.2	31.6 ± 2.9
MDMA (2×/day)	1.15 ± 3.4	3.7 ± 1.9	14.1 ± 3.8	10.9 ± 3.2	6.0 ± 1.1	35.6 ± 4.73
Postnatal						
Saline	0.9 ± 0.07	5.8 ± 0.9	16.6 ± 1.0	9.6 ± 1.1	8.2 ± 1.03	31.7 ± 2.4
MDMA (1×/day)	0.8 ± 0.10	3.8 ± 0.5	9.3 ± 0.7*	7.7 ± 0.9	6.2 ± 1.4	30.2 ± 2.6
MDMA (2×/day)	0.8 ± 0.06	3.0 ± 0.3*	8.5 ± 0.9*	8.5 ± 0.81	5.5 ± 0.73	15.7 ± 1.9*

* Abbreviations: NE, norepinephrine; DA, dopamine; HVA, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid.
* Significant Dunnetts *t* comparisons ($P < .05$) with the saline control group.

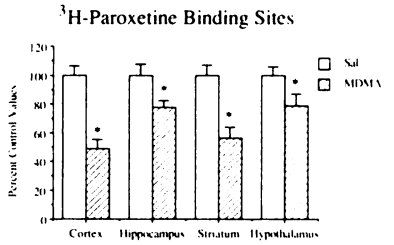

Fig. 6. Effects of repeated systemic administration of MDMA on the density of [^{3}H]paroxetine binding sites in the cortex, hippocampus, striatum and hypothalamus of the postnatally treated pups described in figure 3. Data are plotted as a percentage of the [^{3}H]paroxetine density of control pups and represent the mean ± 1 S.E.M. for eight to nine pups per group. Control values are as follows: cortex, 428 ± 27; hippocampus, 210 ± 16; striatum, 477 ± 33; and hypothalamus, 813 ± 40 fmol/mg protein. Vertical lines at each column represent 1 S.E.M. * Significant independent *t* test comparisons of MDMA with saline control within each brain region ($P < .05$).

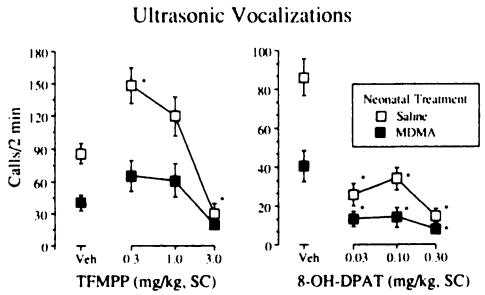

Fig. 7. Acute effects of TFMPP (0.3–3.0 mg/kg, s.c.) and 8-OH-DPAT (0.03–0.3 mg/kg, s.c.) on the rate of ultrasonic calling by 7 to 11-day-old rat pups measured during 2 min social isolation beginning 60 min after injection. Each pup was used twice with a 3-day recovery period between injections. The sequence of dose administration was systematically varied across four litters. Beginning within 12 hr after birth, each pup was injected with saline ($n = 63$; □) or MDMA ($n = 49$; 10 mg/kg, s.c.; ■) at 12-hr intervals up to seven injections. Vertical lines at each data point represent ± 1 S.E.M. * Significant Dunnett's *t* comparisons of dose of TFMPP or 8-OH-DPAT with saline within a neonatal treatment group ($P < .05$), in the presence of significant overall treatment effects detected by analysis of variance.

transmitters and neuromodulators that have not been assayed may also be changing during this period.

The increased rate of calling measured 12 hours after MDMA administration appears to be coincident with the development of re-uptake inhibitor-sensitive neurotoxicity (Schmidt, 1987) or relative depletion. Interestingly, administration of clomipramine has also been associated with an ultrasonic call rate enhancing phase measured 11 hr after the last injection, although this effect probably depends on accumulation of a noradrenergic metabolite (Winslow and Insel, 1990). The excitatory effects of MDMA on ultrasonic calling measured 10 to 24 hr after injection are most consistent with long-term serotonergic depletion, since measures of brain catecholamine levels in adult rats appear to return to near basal levels within 10 hr after administration of 10 mg/kg MDMA (Yamamoto and Spanos, 1988). The two-phase model of acute MDMA behavioral effects in pups, as well as on brain 5HT in adult rats, is also reminiscent of the subjective reports of recreational users who describe a short-term feeling of euphoria and empathy, followed by a persistent dysphoria 24 to 48 hr after use (Peroutka *et al.*, 1988).

Repeated administration of MDMA to adult rats has been shown to produce selective, long-lasting changes in 5HT, 5HIAA and 5HT binding sites in brain (Battaglia *et al.*, 1988). In the studies reported here, repeated administration of a large dose of MDMA to infant rats produced a long-lasting decrease in the rate of calling evident within 5 days of the last injection. Unfortunately, this behavior normally disappears by postnatal day 15 to 17, so more persistent effects of MDMA on calling could not be monitored. Ongoing studies suggest that neonatal administration of MDMA may also be associated with decreased ultrasonic vocalization during aggressive encounters between adult rats (author's unpublished data). But, even within the pre-weaning phase, the decreases in vocalization are surprisingly discrete, with several other developmental measures unaffected. Moreover, these selective behavioral effects do appear to be related to the serotonergic lesion. Not only were cortical levels of catecholamines (norepinephrine, dopamine) and catecholamine metabolites (homovanillic acid, 3,4-dihydroxyphenylacetic acid) unaffected by MDMA (consistent with findings in adult rats), but animals with more modest serotonergic lesions (produced by the lower cumulative dose of MDMA) showed less of a change in vocalization, suggesting a dose-response relationship.

The failure of prenatal administration of MDMA to affect ultrasonic calling or to modify measures of cortical 5HT may reflect the relatively late development of a functional re-uptake site. Indeed, functional re-uptake sites required to mediate MDMA-induced toxicity may not develop until soon after parturition. For example, D'Amato *et al.* (1987) describe a transient, dense pattern of [^{3}H]citalopram binding that could not be detected earlier than postnatal day 2, and then becomes quite prominent between days 7 and 12. Binding becomes more diffuse at 20 days and older. Evidence of developmentally determined "critical" periods for pharmacological effects on

fetal and postnatal brain development is well documented (Lauder and Krebs, 1986; Yanai, 1984). It may also be relevant that although serotonin is present in significant concentrations beginning at embryonic day 13, early 5HT appears primarily to modulate the developmental transition from cell proliferation to differentiation (Lauder and Krebs, 1978, 1986), rather than functioning as a classic neurotransmitter.

Previous efforts to describe long-term behavioral effects related to MDMA neurotoxicity in adult animals have been surprisingly unsuccessful (Nencini *et al.*, 1988; Peroutka *et al.*, 1988; Insel *et al.*, 1989; Wing *et al.*, 1989; Hata *et al.*, 1989), in view of the convincing evidence of serotonergic modulation of "emotional" behavior. The current findings are, however, consistent with the behavioral consequences of neonatal administration of other 5HT neurotoxins. For example, Hård *et al.*, (1982) reported that ultrasonic vocalizations were virtually eliminated by a single injection of 5,7-DHT (25 μg/kg, i.c.v.) associated with a 50% depletion of whole-brain 5HT. Similar effects were found after multiple injections of the 5HT synthesis inhibitor para-chlorophenylalanine (100 mg/kg, route unspecified). Furthermore, decreased calling following para-chlorophenylalanine was reversed by 5-hydroxytryptophan. Selective depletion of serotonin by 5,7-DHT has also been implicated in the modulation of nipple attachment behavior depending on the age of the pup (Leshem and Kreider, 1987). The latency to nipple attachment by suckling pups (9–20 days postnatal) was transiently increased following 5HT depletion, but was significantly decreased when measured at weaning age (21–37 days postnatal). These data demonstrate a consistent role for brain 5HT in the maintenance of mother-infant attachment, and further suggest that this modulatory influence may change during postnatal development (Enters and Spear, 1988).

How then does serotonin modulate infant rat ultrasonic vocalization? The data from this as well as previous studies suggest that both increased synaptic serotonin (acute MDMA, clomipramine, $5HT_{1A}$ and $5HT_2$ agonists) and decreased serotonin (chronic MDMA, 5,7-DHT) reduce ultrasonic vocalization. This paradox is not easily explained, although our data do suggest several pathways that may lead to similar behavioral effects for acute and chronic treatment.

One possible explanation of the paradoxical behavioral effects of MDMA is suggested by the varying effects of 5HT receptor agonists. Our data demonstrate that stimulation of 5HT may inhibit or facilitate ultrasonic vocal behavior depending on which receptor subsystem is involved. 8-OH-DPAT and DOI, relatively selective $5HT_{1A}$ and $5HT_2$ receptor agonists, respectively, significantly reduced the rate of calling, whereas TFMPP, a $5HT_{1B}$ receptor agonist, significantly increased the rate of calling by pups. Similar opposing effects of $5HT_{1A}$ and $5HT_{1B}$ receptor agonists have been previously reported on thermoregulation (Wozniak *et al.*, 1988; 1989), penile erection (Berendson and Broekkamp, 1987) and food intake (Blundell, 1977; Rowland and Carlton, 1986; Dourish *et al.*, 1985, 1988, 1989; Neill and Cooper, 1988) in adult rats. Presumably, the physiological effects of 5HT on each of these measures depends on the interaction between receptor subtype activation. In the current experiments, the acute effects of MDMA resemble $5HT_{1A}$ or $5HT_2$ agonists, consistent with the findings of competitive binding experiments (Battaglia *et al.*, 1988). Repeated administration of MDMA, which destroys 5HT terminals, might selectively reduce $5HT_{1B}$ receptors, many of which are located presynaptically on serotonin terminals (Engel *et al.*, 1986; Raiteri *et al.*, 1986; Conn and Sanders-Bush, 1987). As a result, the interaction of rate increasing and decreasing effects of 5HT activation might be altered in favor of the rate-decreasing effects in MDMA-treated pups. Evidence in support of this formulation is suggested by the reduced efficacy of the $5HT_{1B}$ agonist TFMPP associated with MDMA neurotoxicity (fig. 7). In addition, the high concentration of $5HT_{1B}$ sites in the striatum (Pazos and Palacios, 1985; Vergé *et al.*, 1986) coincides with a region of intense MDMA neurotoxicity (fig. 6).

An alternate possibility depends on the development of supersensitivity of postsynaptic 5HT receptors. The long-term development of supersensitivity to 5HT agonists, particularly $5HT_{1A}$ or $5HT_2$ agonists, has previously been proposed following administration of 5,7-DHT to both infant and adult rats (Pranzatelli *et al.*, 1989; Towle *et al.*, 1984; Pranzatelli and Snodgrass, 1986; Breese *et al.*, 1978). In the current experiments, the failure to detect MDMA-induced changes in behavior until postnatal day 9 (5 days after the last MDMA injection) might reflect the progressive development of supersensitivity. However, if 5HT supersensitivity is involved in the mediation of decreased calling following MDMA treatment, one might expect increased sensitivity to the rate-reducing effects of 8-OH-DAT or DOI. No such increased sensitivity was measured, however, the low baseline rate of calling in the MDMA-treated pups may have masked further decreases.

Finally, neonatal MDMA administration may disrupt a normal developmental process that requires serotonin as a trophic or modulating factor. In fact, MDMA's long-term effects on vocal behavior (fig. 3) resemble the absence of a developmental increase as much as a suppression of vocalization. Others have postulated a trophic role of serotonin on brain development (Lauder and Krebs, 1978, 1986; D'Amato *et al.*, 1987; Blue and Moliver, 1989). However, it is not at all clear how such a trophic effect on neural development could be expressed in the highly specialized behavior of vocalization.

In summary, MDMA produces long-lasting changes in the rate of ultrasonic calling emitted by rat pups at doses that have no measurable effect on motor activity. The direction of change depends on dose, time since injection and the frequency of drug administration. Changes in behavior following repeated MDMA administration are associated with selective depletions of 5HT, probably as a consequence of neurotoxicity. Finally, $5HT_{1A}$ and $5HT_2$ drugs reduced ultrasonic calling in pups independent of neonatal MDMA treatment, whereas rate-increasing effects of a $5HT_{1B}$ drug were blocked by neonatal MDMA treatment. These data demonstrate an important role for 5HT in the modulation of the infant rat's response to maternal separation and consequently in the maintenance of mother-infant attachment. Serotonin has been traditionally assigned a prominent position in the modulation of the dispersive strategies of both primate and rodent species (Raleigh *et al.*, 1980; McGuire and Raleigh, 1987; Olivier *et al.*, 1987). These data suggest that, at least in the early stages of development, serotonin may also be involved in the critical, cohesive mother-infant behaviors.

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