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Research report

Developmental 3,4-methylenedioxymethamphetamine (MDMA) impairs sequential and spatial but not cued learning independent of growth, litter effects or injection stress

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Abstract

Previously, we have shown that rats administered MDMA from postnatal (P) days 11–20 had reductions in body weight during the period of treatment and as adults they had deficits in sequential and spatial learning and memory. In the present study, to control for weight reductions, we used litters with double the number of offspring to induce growth restriction comparable to that of standard size litters treated with MDMA. Litters were treated twice daily from P11 to 20 with vehicle or MDMA (20 mg/kg) or only weighed. Males, but not females, exposed to MDMA had longer latencies and more errors in the Cincinnati water maze compared to males of the other treatments. In the Morris water maze (210 cm pool, 10×10 cm platform), the MDMA animals were impaired relative to all other treatments during acquisition. Only the MDMA females showed deficits when the platform was shifted to a new location, however, both MDMA males and females were impaired when the location of the platform was again shifted and a reduced platform (5×5 cm) used. No differences were observed in the ability to swim a straight channel, locate a platform with a cue, or the endocrine response to forced swim among the treatment groups. No differences were seen between animals injected with saline and those only weighed. The data suggest that factors, such as growth retardation, multiple injections, or the composition of the litter, do not affect the development of learning and memory impairments resulting from P11 to 20 MDMA exposure. The large litter approach offers a novel method to control for undernutrition during the preweaning period in rodents.

Theme: Neural basis of behavior

Topic: Drugs of abuse: amphetamine and other stimulants

Keywords: Amphetamine; Cincinnati maze; Corticosterone; MDMA; Morris maze; Sequential learning and memory; Spatial learning and memory

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA) has become a popular drug of abuse among young adults [10,29]. Because the majority of women MDMA users are of childbearing age, there is concern about fetal exposure [12]. Effects of MDMA in adults include chronic memory disorders, alterations in serotonin systems, and increased cortisol [28,29,36]. Few data exist detailing the consequences of MDMA exposure in utero on human development. One limited study indicated MDMA use during pregnancy increased risk of congenital anomalies, including heart defects and club foot [23]. Similar to the lack of

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human data regarding early developmental exposure to MDMA, there are limited animal data as well.

Developmental exposure to MDMA in rats from postnatal day (P)11–20, a period that correlates with human third trimester hippocampal development [2], induces long-term impairments in both spatial and sequential learning and memory when these animals are adults [5]. Substituted amphetamines are known to be anorectic agents in adult animals and corroborating this effect, the neonatal animals showed reductions in body weight during MDMA exposure [5]. Therefore, nutrition presents a potential confound for the learning deficits produced by neonatal MDMA. This issue is not unique to MDMA or to developmental stimulant exposures generally, but rather has been a long-standing problem in studies that cause growth deficiencies during the preweaning period when the use of pair-feeding techniques are not feasible. While it is reassuring that others have demonstrated that no differences exist in the ability of adult animals to navigate in the Morris water maze when animals are subjected to undernutrition through the use of special diets during development [18,35], this does not entirely alleviate the situation [8]. It is unlikely that the nourishment provided by the dam to the pups was affected since MDMA was administered directly to the pups [5]. The decrease in body weight was more likely the result of reduced feeding or increased metabolism in the offspring. In order to account for the influence of body weight decrements induced by MDMA, we introduced a novel control procedure using increased litter size. Pilot work showed that body weights of litters containing 16 offspring were comparable to animals exposed to MDMA. Two other interpretational concerns arose from our previous study [5]. First, dams may have interacted differentially with pups that received MDMA relative to those given vehicle since we used a within litter design previously. Second, multiple daily injections, a recognized stressor in adult animals, may have effects of its own.

Therefore, the goals of this study were to: (1) determine if the anorectic effect of MDMA administration from P11 to 20 accounted for the learning and memory deficits by using a large litter control group; (2) eliminate differential within-litter maternal responses by employing a between-litter design; (3) include an untreated, weighed only, group to control for multiple injections; and (4) determine if MDMA exposure produced lasting changes in pituitary–adrenal responsiveness prior to and following a forced swim (FS) test.

2. Materials and methods

2.1. Subjects and treatments

Nulliparous female and male Sprague–Dawley CD IGS rats (Charles River Laboratories, Raleigh, NC, USA) were mated in hanging wire cages following an acclimation period to the vivarium of at least 1 week. The day a sperm plug was detected was considered embryonic day 0 (EO). Fourteen days after being placed with the males, the females were transferred to polycarbonate cages (45.7×23.8×20.3 cm). The offspring were the test subjects for the experiment. The vivarium was maintained on a 14:10-h light/dark cycle (lights on at 06:00 h) and food and water were freely available. All procedures were approved by the Cincinnati Children's Hospital Research Foundation's Laboratory Animal Care and Use Committee and the vivarium was fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Beginning on E21, pregnant females were checked twice daily for the presence of a litter. The day of birth was designated as postnatal day 0 (P0). Litters were undisturbed until P1 when the litter was sexed, weighed, and randomly culled to eight pups (four males and four females). When necessary up to two same-age pups were fostered on P1 ($n=14$ litters) to produce an equal distribution of sex or a litter size of eight. The litters were quasi-randomly assigned, with the stipulation that litters were equally distributed to one of four treatments that started on postnatal day (P) 11 and ended on P20. This was a between litter design; all animals in a litter were treated identically with one of the following treatments: (1) 20 mg/kg MDMA 2× per day in a litter size of eight (MDMA); (2) saline 2× per day in a litter size of eight (SAL); (3) saline 2× per day in a litter size of 16 (LARGE), and (4) weighed only 2× per day in a litter size of eight (WEIGHED). There were 15 litters per group for a total of 60 litters.

2.2. Fostering

In order to control for the decrease in body weight produced during the period of MDMA exposure that we had observed previously [5], the LARGE litters were constructed and maintained during the period of treatment only (i.e. 16 pups in a litter from P11 to 20 and eight pups prior to and following drug administration). Because the introduction of non-biologically related pups in the LARGE litters presented the potential that maternal behavior in these litters would be altered, regardless of treatment, all pups from a litter were fostered to lactating dams on P11. Pups that were being treated were identified with an ear punch on P11 and only biological littermates were treated in the LARGE litters.

2.3. Drug and treatment administration

D,L-Methylenedioxymethamphetamine HCL (NIDA) at a dose of 20 mg/kg body weight (expressed as the freebase) or saline vehicle was administered two times daily at 8-h intervals from P11–P20. This dose of MDMA was select-

ed based on previous data that showed it induces impaired learning and memory in the Morris water maze (MWM) as well as the Cincinnati multiple-T water maze (CWM) [5]. Injections were delivered subcutaneously in the dorsum in a volume of 3 ml/kg per injection with injection sites rotated to minimize aggravation to the dermis. No necrotic lesions in the dermis were produced using this method. Prior to each injection the animals were weighed and also weighed weekly thereafter. A control group that was only weighed at each of the treatment times was included to assess if the multiple dose regimen affected the ability of the animals to learn. Following treatment, on P21 the LARGE litters were reduced to eight pups. All pups were naturally weaned [3,32] and separated from their foster mothers on P28 in same sex pairs and maintained in polycarbonate cages. Only two males and two females per litter were used for the behavioral tests. Each of these animals was tested in all of the procedures and the behavioral results were averaged for the males as well as the females and sex was then treated as a within-litter factor. The other animals in the litter were given to other investigators.

2.4. Behavioral tests

2.4.1. Straight channel

On P50, prior to any tasks of learning and memory, the motor/swimming capability of the animals was assessed in a 244 cm long×15 cm wide channel. The channel was filled to approximately 35 cm with room temperature water (21±1 °C). Each animal was placed in the tank facing one end wall and allowed to swim for up to 2 min to find an escape ramp that was located at the opposite end. Four consecutive trials were administered to each animal and the latency to reach the escape ramp was recorded for each trial.

2.4.2. Cincinnati multiple-T water maze

One day after the straight channel, animals were tested in the Cincinnati multiple-T water maze (CWM). The CWM was made of black acrylic with nine conjoined Ts that formed a central channel as described previously [37,39]. The apparatus was placed in a large tank of water (temperature 21±1 °C). Rats were administered two trials per day, with a maximum of 5 min per trial, for 5 days (path B; intertrial interval (ITI)=30 min when animals failed to escape in the allotted 5-min time period). Since assistance does not appear to influence learning in this task, the rats were not assisted to the escape ramp if they failed in the 5-min time period, rather they were removed directly from the maze and placed in their home cage until the next trial [39]. The dependent measures were errors of commission (whole-body entries into cul-de-sacs of the Ts) and latency to escape. In summary, the CWM task requires the animal to learn a sequence of turns within a central channel in order to reach an escape ramp. Although the neuroanatomical basis for learning in the CWM is unknown, learning in both a water and a dry version has been shown to be differentially affected by drug regimens when compared to MWM learning [31,38,41,42]. Since the MWM tests spatial learning and memory [26] it appears that the CWM measures a different type of learning (i.e. sequential) or a combination of sequential and spatial learning. The CWM and the straight channel were located in the same room.

2.4.3. Morris water maze

2.4.3.1. Apparatus. The Morris water maze apparatus was 210 cm in diameter with black walls and filled with water to a level that was 2 cm higher than the platform that measured 30.5 cm high. Two different sized platforms were used depending upon the task requirements. The larger platform was 10×10 cm and the reduced size was 5×5 cm. The platforms were made of a clear acrylic with nylon screen glued to the top to increase traction. Several prominent extra-maze cues were placed on the walls in the room as well as curtains that were fastened together at opposite ends of the maze. Cardinal positions used as start locations were arbitrarily designated as north, south, east, or west, with the north position located furthest from the experimenter. The platform position was counterbalanced and placed in either the southeast or northwest quadrant and animals were started from one of the four cardinal positions in a quasi-random order, such that all four positions were used each day. A camera was positioned over the middle of the maze and attached to a computer with the Poly-Track video system (San Diego Instruments, San Diego, CA, USA). The latency as well as a trace of each animal's swim path was obtained for each trial.

2.4.3.2. Acquisition. The acquisition phase started on P57 and lasted for 6 days. The animals received four learning trials on each day with a maximum of 2 min per trial. The platform for the acquisition phase was 10×10 cm and the ITI was 30 s beginning when the animal reached the platform. If an animal did not find the platform in the allotted 2 min, it was removed from the water and placed on the platform for 30 s. The dependent measures for the hidden platform version of this task were the latency and path length to reach the platform and the cumulative distance from the platform.

Two probe trials were performed with the platform removed: one prior to the learning trials on day 3 of testing and one following the training trials on day 6. For the 30-s probe trial, the platform was removed and the animal was started at a novel position that bisected two of the cardinal start positions. Dependent measures for the probe trial included average distance from the target and the number of target site crossings. Every 55 ms the distance from the platform was calculated for each animal and then averaged over the 30-s trial to obtain the average distance measure.

A tally of each time the animal eclipsed the area where the platform had been located was used for the target site crossing measure.

2.4.3.3. Shifted position. Starting on P64 the 10×10 cm platform was shifted 180° to a new quadrant of the maze, such that the animals that originally had the platform located in the SE quadrant now had the platform in the NW quadrant and vice versa. The same procedure and dependent measures used for the acquisition phase were employed during this phase of testing.

2.4.3.4. Shifted position, reduced platform. Starting on P71, animals were tested with the platform shifted back to the original platform location used during acquisition, however, the platform size was reduced to 5×5 cm. Animals were tested using the same protocol and measures as before.

2.4.3.5. Cued learning. Beginning on P78, animals were tested for cued platform learning to determine if any differences in the MWM could be attributed to either an inability to use cues to locate the hidden platform or a difference in motivation to escape the water. The cued platform had a cylindrical, white marker mounted on a brass rod 12 cm above the surface of the water. The platform surface was maintained 2 cm under the water. The white curtains were closed around the exterior of the maze, thereby removing a majority of the extramaze cues (overhead cues were still available). Cued platform testing was administered for 6 days with four trials per day. Platform and start positions were randomized over the trials and the start position for each trial was from one of the four cardinal positions. The latency to find the platform was recorded manually for each trial using a stopwatch, and animals were given 2 min to locate the platform. If the animals did not find the platform, they were placed on it. All animals had 30 s on the platform plus 20–30 s in a holding cage while the platform was moved to its new location.

2.5. Forced swim (FS)

The hormonal response of the animals to a forced swim was assessed at the end of behavioral testing ($n=12$ litters in each treatment). Within 2 weeks from the last measurement of weight (P91), animals were placed in a 15.2 cm diameter by 45.7 cm tall cylinder filled with 30 cm of room temperature water (21±1 °C) for 15 min. One male and one female from each litter received the forced swim and were immediately decapitated upon removal from the water, while another male and female pair were removed directly from the home cage and decapitated.

2.6. Hormone assessment

Trunk blood was collected in ice-chilled polypropylene tubes containing EDTA. Blood was centrifuged at 2400 rpm for 15 min at 4 °C and plasma collected, aliquoted, and stored at −80 °C until the hormones were assessed. To protect against ultradian or circadian variations in hormone concentrations [15,44], blood samples were collected within a 2-h period (11:00–13:00 h).

Commercially available [^{125}I]RIA kits were used to determine hormone concentrations in duplicate plasma samples. Corticosterone (CORT) kits were purchased from ICN Biomedicals (Costa Mesa, CA, USA). The plasma samples for CORT were diluted 1:200 prior to the assay. ACTH kits were obtained from Diagnostic Products (Los Angeles, CA, USA). Inter- and intra-assay variability was under 10%.

2.7. Statistics

Analysis of variance (ANOVA) was used to determine group differences. When animals were tested over multiple days and trials or under conditions of stress (swim and no swim), the data were analyzed with day and trial or stress as within subject variables. Furthermore, the litter was the unit of analysis and sex was considered a within variable for all analyses [1,14]. For analyses that had within-subject variables, tests of sphericity were conducted and when these tests failed, Greenhouse–Geisser corrections were applied. Tests of simple effects were used to analyze significant interactions. Significant main effects were analyzed further to determine group differences using the step-down F-test procedure of Kirk that maintains α at $P<0.05$ for all a posteriori comparisons [16]. A $P\leq 0.05$ was used to determine significance. In order to maintain clarity in the presentation of results, only the F values for the significant main and interaction effects that were treatment or sex related are reported. Most other significant effects are only listed in the text.

3. Results

3.1. Body weights

An ANOVA of body weights prior to treatment (P1 and P7) revealed only a Sex difference, $F_{(1,56)}=42.86$, $P<0.0001$, where males weighed more than females. A separate ANOVA with Treatment, Sex, Day, and Time (am or pm) as the main effects was performed on body weights during the P11–20 period of treatment. The main effects of Treatment, $F_{(3,56)}=21.20$, Sex, $F_{(1,56)}=64.71$, Day, and Time were significant ($P<0.0001$ for all effects). As expected, regardless of treatment, animals gained weight over the treatment period, and males (Fig. 1A) weighed more than females (Fig. 1B). The highest order interaction with Treatment was Treatment×Day×Time, $F_{(27,504)}=3.62$, $P<0.0001$. Simple effects and step-down analysis demonstrated that no differences in body weights were apparent on P11 or P12, however, differences were noted

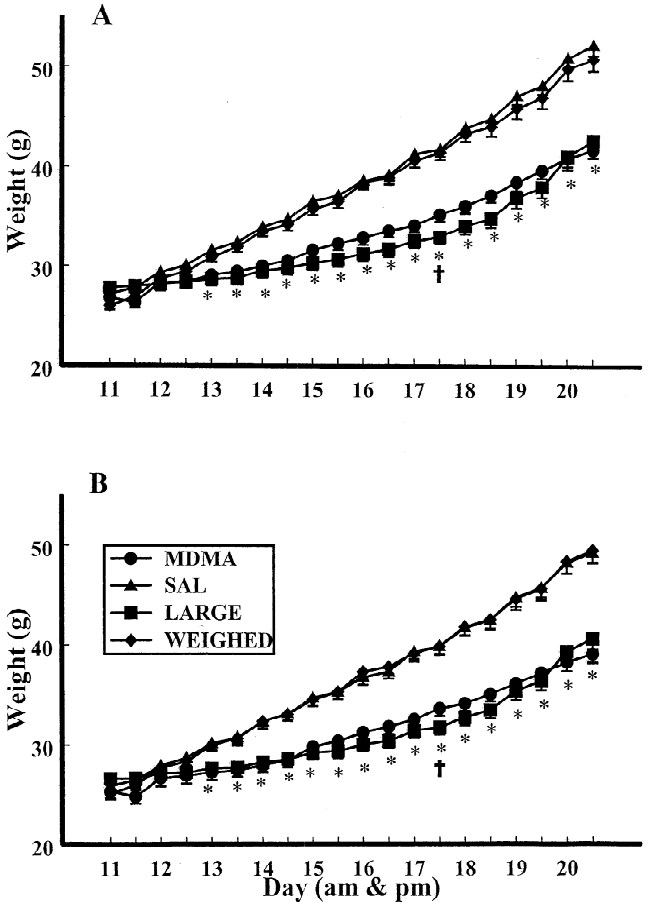

Fig. 1. Body weights of pups, males panel A and females panel B, from postnatal day (P) 11–20 following administration of 20 mg/kg MDMA (MDMA), saline (SAL), or only weighed (WEIGHED) twice daily in a litter size of eight or raised in a litter of 16 pups and administered saline twice daily (LARGE). The animals from the MDMA litters and LARGE litters were lighter than animals from either the SAL or WEIGHED litters starting on P13 and continuing throughout the dosing period. With the exception of the P17 afternoon weight,, in which the LARGE litters weighed less than the MDMA, no differences were observed between these two treatments in body weight. No differences were observed between the SAL and WEIGHED groups. Males weighed more than the females. * $P<0.05$, MDMA or LARGE compared to SAL or WEIGHED. † $P<0.05$ MDMA versus LARGE.

from P13 to 20. From P13 to 20, the MDMA and LARGE litters had smaller body weights than either the SAL or WEIGHED litters, regardless of Day or Time, $P<0.01$. With the exception of the afternoon weight on P17, no differences were noted between the MDMA and LARGE litters, and no differences were observed between the SAL and WEIGHED litters (Fig. 1).

The weekly body weights of animals after the treatment period were analyzed in two separate ANOVAs, the weights prior to separation from mothers (P21 and 28) and those after separation (P35–91). Prior to separation, the main effects of Treatment, $F_{(3,56)}=18.29$, Sex, $F_{(1,56)}=112.24$, and Day were significant, $P<0.0001$. The step-down analysis showed that, similar to the dosing weights, the MDMA and LARGE treatments had smaller body weights than the SAL and WEIGHED treatments (not shown). No differences were found between the MDMA and LARGE groups or between the SAL and WEIGHED groups on these days. No interactions with Treatment were observed.

No Treatment main effect or any interactions with Treatment were found for the body weights from P35 to 91. Significant effects of Sex, $F_{(1,56)}=2597.8$ and Day were found, $P<0.0001$. The males weighed more than the females and animals increased in body weight during each week.

3.2. Straight channel and Cincinnati multiple-T water maze

No differences in Treatment or Sex were noted in the straight channel swimming latencies of the animals (Table 1), although animals improved their escape time over the four trial test (Trial, $F_{(3,168)}=126.11$, $P<0.0001$).

The data for the latency and number of errors to escape the CWM were not normally distributed and therefore were subjected to a log (mean+1) transformation prior to the ANOVA (Treatment, Sex, Day, Trial). For latency, no Treatment main effect was observed, however, Treatment×Sex, $F_{(3,56)}=4.51$, $P<0.007$, Treatment×Sex×Day, $F_{(12,224)}=2.10$, $P<0.02$, and Treatment×Trial, $F_{(3,56)}=3.26$, $P<0.03$, interactions were found (Fig. 2A,C). The interactions revealed that the males (Fig. 2A) treated with MDMA had longer escape latencies than males in the other conditions. These differences in latency were most pronounced on day 4 and 5 of testing when comparing the MDMA animals to the other three treatments, although on day 5 no difference was noted between the male MDMA and the male WEIGHED animals (Fig. 2A). No differences were noted among the females (Fig. 2C). Post-hoc analysis of the Treatment×Trial interaction was not significant. The main effects of Day and Trial as well as the interactions of Sex×Day and Day×Trial were also significant.

The number of errors (entering a dead-end cul de sac) was counted for each trial and significant interactions were observed: Treatment×Sex, $F_{(1,56)}=2.70$, $P=0.05$ and Treatment×Trial, $F_{(3,56)}=3.42$, $P<0.03$. Analysis of the interactions showed that the males treated with MDMA committed more errors than males in the other treatments (Fig. 2B). No differences were detected among the females (Fig. 2D) or the further analysis of the Treatment×Trials interaction. The main effects of Day and Trial, and the Day×Trial interaction were significant. The Treatment main effect approached significance as did the Treatment×Sex×Day and Treatment×Sex×Trial interactions, $P<0.10$.

3.3. Morris water maze

3.3.1. Acquisition

Separate ANOVAs (Treatment, Platform position, Sex,

Table 1
Straight channel, Morris maze probe and visible platform data from animals administered either MDMA (litter size=8), saline (SAL, litter size=8), saline (LARGE, litter size=16) or were only weighed (WEIGHED, litter size=8) from postnatal day 11–20 (mean±S.E.M. are presented)

Behavior	MDMA	SAL	LARGE	WEIGHED
Straight channel (s)	16.30±0.79	16.99±0.80	17.88±1.08	17.60±1.36
Morris acquisition, probe				
Average distance (cm)	73.86±1.52**	66.97±1.92	69.83±2.07	68.34±2.57
Crosses	0.74±0.07†	1.12±0.08	1.02±0.10	0.85±0.09
Morris shifted, probe				
Average distance (cm)	65.25±1.55	63.36±1.38	66.83±1.29‡	61.94±1.29
Crosses	1.13±0.11	1.36±0.14	1.04±0.10‡	1.22±0.12
Morris shifted, reduced probe				
Average distance (cm)	66.52±1.57*	59.86±1.24	59.98±1.53	60.41±1.74
Crosses	1.22±0.10	1.42±0.12	1.53±0.12	1.69±0.19
Morris cued (s)				
Males*	12.95±0.97	11.69±0.72	12.56±1.24	11.16±0.71
Females	18.29±1.10	14.40±0.79	16.34±1.26	15.40±0.88

* $P<0.05$ MDMA vs. other treatments and male vs. female. ** $P<0.05$ MDMA vs. SAL and WEIGHED. † $P<0.05$ MDMA vs. SAL. ‡ $P<0.05$ LARGE vs. WEIGHED, see text for explanation of interaction.

Day, Trial) were performed on latency and path length to locate the platform as well as the cumulative distance from the platform during the acquisition phase. For the latency to locate the platform (Fig. 3A), the main effects of Treatment, $F_{(3,52)}=4.87$, $P<0.005$, Sex, $F_{(1,52)}=27.04$, $P<0.0001$, and Day and Trial were significant. The MDMA-treated animals had longer latencies than animals in the other treatments (Fig. 3A). Males found the platform quicker than females (not shown). No interactions with Treatment were found. There was a Day×Trial interaction. For path length (Fig. 3D), the main effects of Treatment, $F_{(3,52)}=3.92$, $P<0.02$, Sex, $F_{(1,52)}=34.03$, $P<0.0001$, and Day and Trial were significant. Similar to latency, longer path lengths were observed for the MDMA animals relative to animals in the other treatments and males had shorter path lengths. No interactions with Treatment were noted for path length. The Sex×Day×Platform position and Day×Trial interactions were also significant. The ANOVA for cumulative distance (Fig. 3G) demonstrated significant main effects of Treatment, $F_{(3,52)}=5.13$, $P<0.004$, Sex, $F_{(1,52)}=31.70$, $P<0.0001$, Platform position, Day, and Trial. The MDMA-treated animals on average were further from the platform while swimming than the animals in the other treatments (Fig. 3G). No Treatment-related interactions were found. Significant interactions for Day×Platform position and Day×Trial were noted. No differences were detected among the SAL, LARGE, or WEIGHED groups for any of the measures.

As a test of memory, animals were given two probe trials during the acquisition phase. One probe trial was given prior to Day 3 learning trials and one immediately following Day 6 learning trials. The average distance from the platform site showed significant Treatment, $F_{(3,52)}=2.80$, $P<0.05$, Sex, $F_{(1,52)}=24.48$, $P<0.0001$, Platform position, and Trial main effects. For average distance, the MDMA animals were on average further from the target location than either the SAL or WEIGHED animals, but the difference between the MDMA and LARGE animals for this measure fell short of significance (Table 1). No interactions with Treatment were found. The Trial× Platform position and Sex×Trial×Platform position interactions were significant (not shown).

The number of times the animals crossed the location where the platform had previously been located was analyzed. The Treatment, $F_{(3,52)}=3.91$, $P<0.02$ and Trial main effects were significant. No other main effects or interactions were significant. The MDMA animals had fewer platform site crossings than the SAL animals (Table 1).

3.3.2. Shifted position

Animals were tested for the ability to learn a new platform position by shifting the platform 180° from the original platform position. For latency (Fig. 3B), the main effects of Treatment, $F_{(3,52)}=3.06$, $P<0.04$, Sex, $F_{(1,52)}=36.07$, $P<0.0001$, as well as Platform position, Day, and Trial, were significant. The Treatment×Sex interaction was significant, $F_{(3,52)}=3.02$, $P<0.04$. No other Treatment-related interactions were observed. The MDMA animals had longer latencies than the SAL or WEIGHED animals (Fig. 3B), this effect was greatest in the females. There was a trend for the MDMA females to have longer latencies than the LARGE group females, $P=0.09$. In addition, there were Day×Platform position, Trial× Platform position, Sex×Trial, Sex×Trial×Platform position, and Day×Trial interactions. Similar findings were observed for the path length (Fig. 3E) and cumulative distance (Fig. 3H) measures (main effects of Treatment, $F_{(3,52)}=3.22$ and 4.12, $P=0.03$ and 0.01, respectively, Sex, $F_{(1,52)}=36.26$ and 40.64, $P<0.0001$, respectively) and for Platform position, Day, and Trial. Treatment×Sex was significant for both path length and cumulative distance,

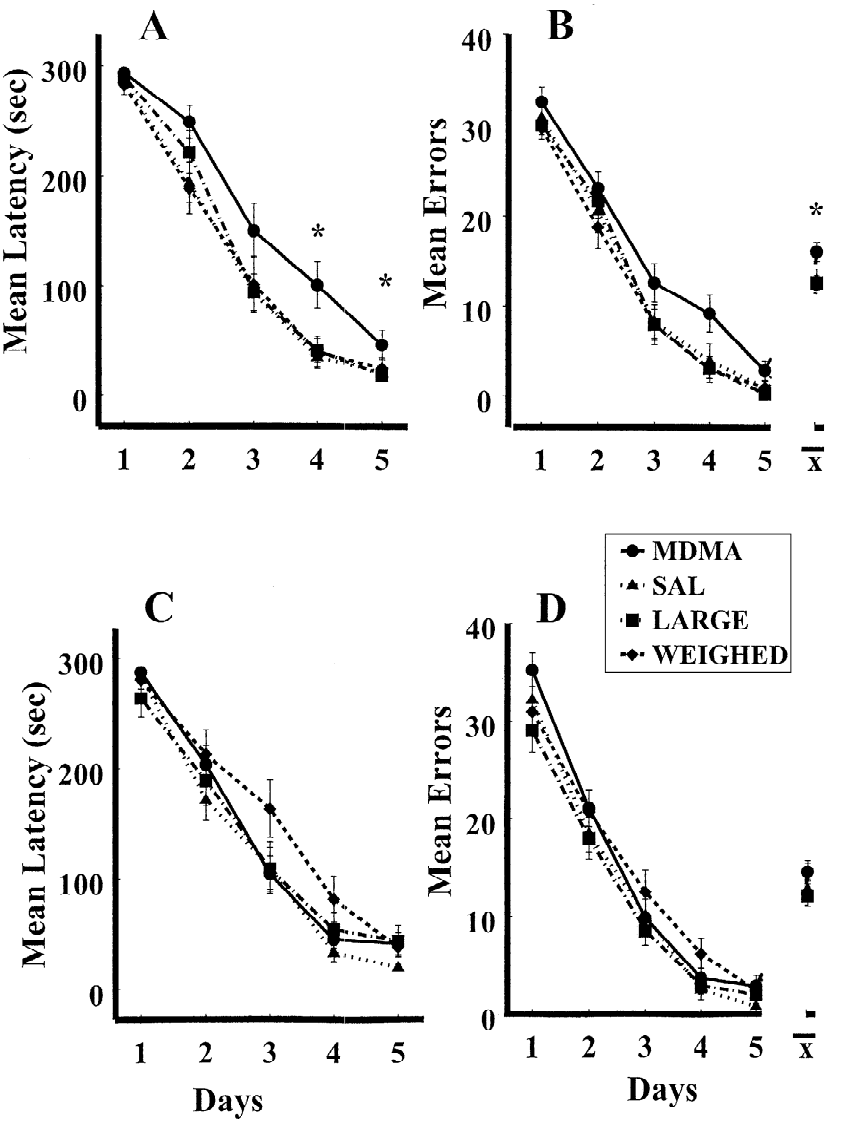

Fig. 2. The mean time per trial (males panel A and females panel C) and the mean number of errors (males panel B and females panel D) on each day in the Cincinnati water maze are represented. For the analysis of these data a log transformation was completed, however raw data are displayed. No treatment main effect was seen for either measure, however an interaction with Sex was shown. Males treated with MDMA had longer latencies on day 4 and 5 of testing (panel A) and more errors (panel B) overall when compared to the males in the other treatments. * $P<0.05$.

$F_{(3,52)}=4.21$ and 4.04, $P<0.02$, respectively. Post hoc analysis showed that the MDMA animals had longer path lengths relative to the SAL and WEIGHED groups (Fig. 3E). This effect was greater in the females than in males. Similar to latency, the MDMA female group's increased path length approached significance relative to the LARGE female group, $P<0.06$. For cumulative distance, the MDMA animals were further from the platform compared to the SAL and WEIGHED groups, an effect that, again, was largest in the females. The MDMA female group was also further from the platform than the LARGE female group (Fig. 3H). Other non-treatment-related interactions for path length and cumulative distance included Day×Platform position, Trial×Platform position, Sex×Trial, Sex×Trial×Platform position, and Day×Trial. No differences were noted among the SAL, LARGE, or WEIGHED groups for any measure.

For average distance in probe trials, there was an effect of Treatment, $F_{(3,52)}=2.79$, $P<0.05$, Sex, $F_{(1,52)}=51.62$, $P<0.0001$, Platform position, and Trial. No group differed significantly from the MDMA group. The only significant comparison was that the LARGE group was further from the platform than the WEIGHED group (Table 1). The interaction of Treatment×Sex×Trial×Platform position was significant, $F_{(3,52)}=2.71$, $P=0.05$. The Sex×Trial×Platform position and Trial×Platform position interactions were also significant. Inspection of the Treatment×Sex×Trial×Platform position suggested that on trial-2 WEIGHED females with the platform in the SE quadrant were closer to the platform than females in the other

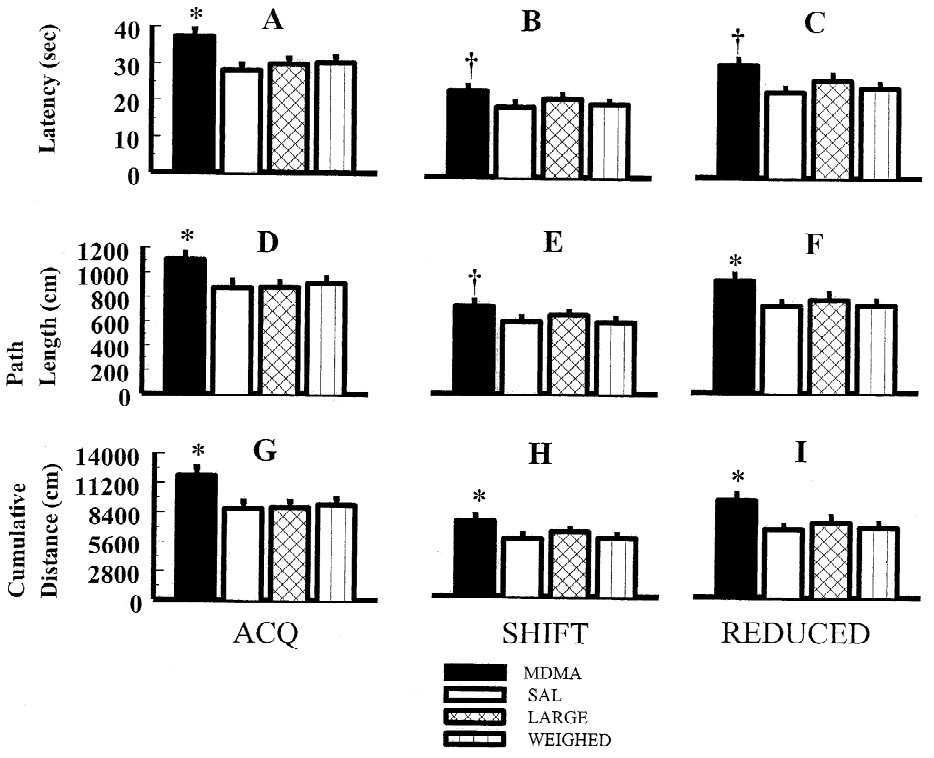

Fig. 3. Performance in the Morris water maze during the learning portion of the acquisition phase (ACQ, panels A, D, G), the shifted platform phase (SHIFT, panels B, E, H) where the platform was located 180° from the original position, and the shifted-reduced (REDUCED, panels C, F, I) phase where a smaller platform (5×5 versus 10×10 cm) was used and the platform was located in the original position. Data represent the mean latency (A,B,C), path length (D,E,F), and the cumulative distance from the platform (G,H,I) for the 24 trials administered. Cumulative distance was measured every 55 ms by the tracking program. In acquisition (A,D,G), the MDMA animals performed worse on all measures relative to all other treatments. In the shifted platform phase (middle column), the main effect of treatment was significant for all measures, but the interaction of sex×treatment was as well. The females from the MDMA treatment performed worse than females in the other treatments for cumulative distance (H) and worse than the SAL or WEIGHED animals on the latency (B) and path length (E) measures. In the REDUCED phase (C,F,I), the MDMA treated animals all performed worse than the animals from the other treatments, regardless of sex. * $P<0.05$ versus SAL, WEIGHED, and LARGE. † $P<0.05$ versus SAL and WEIGHED.

groups. Furthermore, the SAL female group was closer than the MDMA female group.

For the number of platform site crossings, the main effects of Sex, $F_{(1,52)}=5.04$, $P<0.03$, and Trial were significant but the main effect of Treatment was not. However, the interaction of Treatment×Trial×Platform position was significant, $F_{(3,52)}=5.81$, $P<0.002$. Analysis of the Treatment×Trial×Platform position interaction demonstrated that on the last probe trial, the LARGE group that had the platform located in the SE quadrant, had fewer site crosses than any of the other groups (not shown, see Table 1 for overall mean). Sex×Trial×Platform position was also significant. No other differences were detected among the SAL, LARGE, and WEIGHED groups for number of site crosses.

3.3.3. Shifted position, reduced platform

The last hidden platform phase consisted of relocating the platform to the original location used during acquisition with a smaller platform to increase the spatial demands of the task. During this phase of testing some computerized data files were lost due to equipment malfunction, therefore $n=12–14$ per Treatment. The latency to reach the platform was affected by Treatment, $F_{(3,44)}=4.21$, $P=0.01$, Sex, $F_{(1,44)}=17.27$, $P<0.0001$, Day, Trial, and the interactions of Sex×Trial, and Day×Trial. The MDMA animals took longer to locate the platform than animals in the SAL or WEIGHED groups, while the difference between the MDMA and LARGE groups for latency fell short of significance (Fig. 3C). For path length and cumulative distance, Treatment, $F_{(3,44)}=4.23$ and 5.71, respectively, $P\leq0.01$, Sex, $F_{(1,44)}=21.41$ and 22.43, respectively, $P<0.0001$, Day, Trial, and the interactions of Day×Trial, Sex×Trial, and Sex×Trial×Platform position, affected these measures. For cumulative distance, the interaction of Treatment×Sex approached significance, $P<0.08$. Post-hoc comparisons of the Treatment main effects showed that the MDMA group had longer path lengths (Fig. 3F) and were further from the platform (Fig. 3I) during the learning trials than animals in any of the other groups. No differences were noted among the LARGE, SAL, or WEIGHED groups for latency, path length, or cumulative distance.

For the memory trials during this phase, the average distance from the platform showed significant main effects for Treatment, $F_{(3,44)}=4.19$, $P=0.01$, Sex, $F_{(1,44)}=20.94$,

$P<0.0001$, and Trial. The MDMA animals were further from the platform site on average than animals in the other groups (Table 1). No differences were noted among the other groups. No Treatment or Sex main effects were noted for the number of platform site crosses, although the MDMA group had the fewest site crossings (Table 1). The main effect of Trial was significant.

3.3.4. Cued platform

For the cued platform version of the MWM, no differences were noted for the main effect of Treatment or any interaction with Treatment. A Sex difference was noted, $F_{(1,56)}=79.83$, $P<0.0001$, as well as the Sex×Trial interaction, $F_{(3,168)}=8.67$, $P<0.0001$. Similar to the hidden platform version of this task, regardless of treatment, the males found the platform faster than the females and this effect was greater at the start of each day (Table 1). The Day and Trial main effects and the Day×Trial interaction were also significant.

3.4. Hormone concentrations

Total CORT and ACTH were measured in plasma under resting conditions and following 15 min of forced swim. ANOVA (Treatment, Stress, Sex) demonstrated a significant Stress main effect for both CORT and ACTH, $F_{(1,44)}=597.93$ and 255.22, respectively, $P<0.0001$ (Fig. 4), but no effect of Treatment or Treatment-related interactions. Animals that swam for 15 min had higher plasmatic

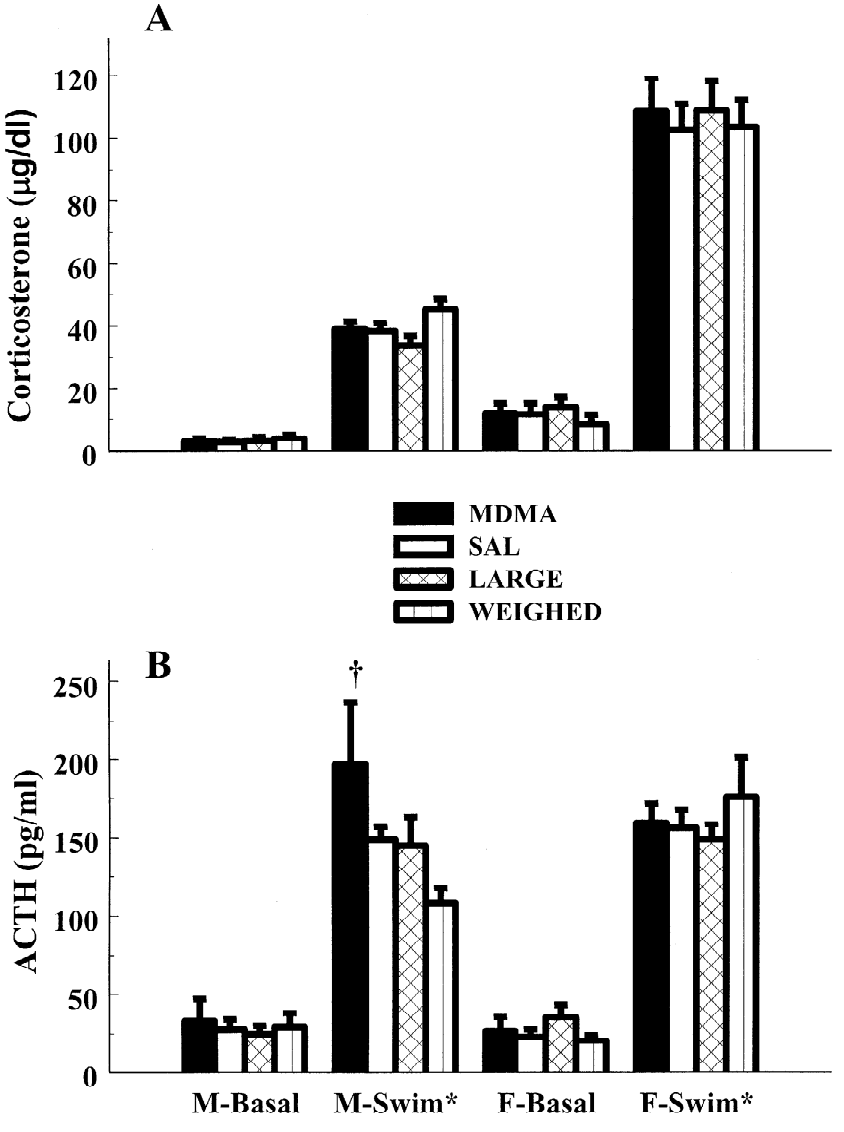

Fig. 4. Plasmatic levels of corticosterone (A) and ACTH (B) in males (M) and females (F) under resting conditions (Basal) or following a 15-min forced swim (Swim). As expected, the FS procedure significantly increased levels of both hormones in the plasma. Females had higher levels than males. No treatment related effects were noted with the exception of the FS-induced release of ACTH in the males exposed to MDMA. * $P<0.05$ versus Basal. † $P<0.08$ versus WEIGHED males.

hormone levels than animals removed directly from the home cage. For CORT, the Sex effect and the interaction of Stress and Sex were significant, $F_{(1,44)}=234.36$ and 151.60, respectively, $P<0.0001$. The females had higher levels of CORT relative to the males under resting and basal conditions. For ACTH concentrations, the interaction of Treatment×Stress×Sex approached significance, $F_{(3,44)}=2.58$, $P<0.07$ and was the result of lower levels in the WEIGHED male group.

4. Discussion

Animals exposed to MDMA on P11–20 were impaired in both sequential and spatial learning and memory, as we had previously demonstrated [5]. In the present study we used a novel control for the body weight reductions produced by MDMA by increasing the size of the litter in one of the control groups. This large-litter procedure produced body weights that were indistinguishable from the body weights of animals exposed to MDMA during the period of drug administration as well as immediately following this period (see Fig. 1A,B). Although body weights were similar between the MDMA and LARGE litters, no apparent learning and memory deficits were observed in the animals from the LARGE litter group. For example, the LARGE litter group had no difficulty solving the CWM. Similarly, the LARGE group showed no difficulty learning the MWM, despite showing some minor differences during the probe trial of the shifted platform phase. These data provide support for the view that early undernutrition does not affect later spatial learning and memory [18,35], and indicate that sequential learning in the CWM is also unaffected by undernutrition. The central point, however, is that the present data show that developmental MDMA-induced anorexia and growth retardation do not produce and do not have a permissive role in the spatial and sequential learning impairments induced by MDMA. Furthermore, the design of the present study offers a new method for use in drug studies to control for growth reductions produced by treatment during the preweaning period of development.

The period of MDMA treatment (P11–20) in the present study has been correlated with hippocampal development in the third trimester of humans. A recent epidemiological study shows that women are more likely to ingest MDMA during the first and second trimester [12]. This study, however, was based upon a population of pregnant women who were seeking guidance on the effects of drug exposure on the developing fetus. Women seeking prenatal care may not be representative of all exposed women. For example, among cocaine users, those women who seek prenatal care tend to stop drug use early in the pregnancy, whereas women who do not seek prenatal care tend to continue drug use throughout pregnancy [33]. If MDMA users show a similar dichotomy, there may be a group of women who continue to expose their fetuses during the third trimester.

In the MWM, we used several indices of spatial learning that included latency to reach the platform, path length, and cumulative distance from the platform. These multiple factors were analyzed since others have argued that some measures of spatial learning and memory in the MWM may be more sensitive or more accurate than others. For instance, latencies may be similar between two groups, although the path to reach the target may be longer or not as direct [19]. Likewise, path lengths may be similar but the cumulative distances from the platform may be different if one group searches farther from the goal [9]. The present data, using all three principal measures, suggest that cumulative distance is slightly more sensitive, at least to the effects induced by developmental MDMA. For example, cumulative distance was the parameter that demonstrated the clearest differences in the spatial learning ability of the MDMA animals compared to the LARGE litter group during all three phases of learning.

We have previously demonstrated that animals exposed to methamphetamine (MA) during the same P11–20 period have deficits in the MWM, however, if the animals are given MWM pretraining prior to acquisition, acquisition differences in spatial navigation are no longer apparent [43]. Nonetheless, the MA animals exhibit spatial and other strategy (e.g. swimming away from the periphery) deficits when a new platform location has to be learned. In the present study, we did not use the random platform pretraining method, however, the animals were given extensive swimming experience in the CWM prior to being placed in the MWM. As suggested recently by others [13] and shown here, swimming experience alone was not enough to attenuate the effects of early MDMA exposure since differences between the MDMA treatment and multiple control groups were apparent during the acquisition phase of the MWM. During the shifted platform phase, however, the MDMA group was still impaired. Parenthetically, this MDMA shifted position learning impairment was largest among the females. When the platform was repositioned a second time and reduced in size, the MDMA spatial navigation impairment was even more pronounced. Collectively, the data suggest that spatial navigation ability, even after extensive training (48 previous learning trials), is persistently altered in the MDMA animals and is probably not a function of sensorimotor deficits in these animals. The lack of treatment effects in both the straight channel and the cued version of the MWM support this interpretation.

Use of a between litter design in this study did not alter the results obtained from our earlier work [5], that is, exposure to MDMA from P11 to 20 produces deficits in the CWM and MWM. Because we did not directly compare the two types of litter designs, we can only make indirect comparisons. The important conclusion is that we

have demonstrated that both designs produce comparable deficits in learning and memory. Concerns over the use of a between litter design versus a within litter design have been raised previously since potential confounds could theoretically exist [4,30]. The findings from these previous studies, however, should be interpreted with caution since group sizes were small and no data were collected after weaning using tests of cognition. One main concern with the use of split-litter designs is the possibility that maternal care will be differentially afforded to treated relative to control animals. The present data alleviate this concern despite the fact that in the LARGE treatment in this study, it is probable that maternal care toward each pup was limited. However, no differences were noted for learning and memory when comparing these animals to the animals from the SAL and WEIGHED groups, where there were only half as many pups. Maternal care has been recognized as an important determinant in the ontogeny of an animal's development, including its response to later environmental challenges (for review see Ref. [7]). The present data, however, show that litter sizes that ranged in number from eight to 16 during the P11–20 period of development did not induce long-term effects on MWM or CWM performance.

We also included a group in this study that was only weighed at each of the time points since we have previously shown that multiple injections of saline on P11 produces a small increase in CORT [40]. No differences were observed in the SAL animals relative to the WEIGHED animals in learning and memory ability or in the endocrine parameters, suggesting that the multiple injection procedure alone did not influence neurobehavioral development of MWM or CWM performance. The HPA axis influences spatial learning and memory [21]. Here we showed that females had a larger response to FS than males and the females, regardless of treatment, performed worse in all aspects of the MWM. Correlation of the male and female cumulative distance from the platform means would suggest that males and females use different strategies to solve the maze. For example, the correlation coefficient for cumulative distance between males and females in the reduced platform phase (similar effects were observed for acquisition and the shifted-platform phases) was only $R^2=0.156$, showing that only a small portion of the variance in female performance is explained by male performance. Hence, males and females use different approaches to solving the maze, a fact that merits further investigation. Of greater importance in this context, however, is that MDMA produced deficits in learning and memory, regardless of gender-specific differences in learning strategies.

Various parameters can affect the development of the HPA axis including sex, but environmental manipulations (i.e. stress, drug administration, or handling) early in development can also affect how the HPA axis responds to stressors later in life. Furthermore, some neurotransmitters such as serotonin (5-HT) appear to play a role in development of the HPA axis. MDMA causes the release of 5-HT and has been shown to slightly reduce the levels of 5-HT in the hippocampus of adult animals exposed to MDMA from either P1–10 or P11–20 [5]. Serotonin and its subsequent metabolism in the hippocampus during early development has been correlated with the number of glucocorticoid receptors (GR) in the adult hippocampus [34]. The effect of 5-HT on GR number appears to be mediated by increased activation of cAMP acting through PKA during development [24]. Glucocorticoid receptors in the hippocampus play an integral role in down-regulating the body's response to stress [11]. Although there are no data showing long-term changes in GR in animals exposed to MDMA early in development, adult animals exposed to MDMA show decreases in GR [20]. Furthermore, adult animals exposed to MDMA and immobilized 7 days later show an attenuated release of 5-HT in the hippocampus and frontal cortex to the immobilization without a concurrent difference in CORT release [22]. In this study, we explored the possibility that neonatal animals given MDMA may have an altered response to stress (i.e. swimming) in adulthood. We collected data for basal and immediate stress-induced levels of CORT and ACTH. No differences were observed in CORT and only a trend was seen for MDMA males relative to the WEIGHED males for ACTH. The lack of differences at these time points does not preclude the possibility that the MDMA animals have a differential response to stress, especially when considering the trend towards elevated levels of ACTH in the MDMA males and the possibility that neurotransmitter changes may have been present without changes in the CORT response. Furthermore, these animals had vast experience in swimming tasks and this may have diminished differences among the groups.

Spatial learning and memory in the MWM has been shown to be dependent upon an intact hippocampus [27]; however, the neuroanatomical regions important for learning in the CWM have not been characterized. Since the ability to process sequential information has been shown to involve the frontal cortex and in particular the prefrontal cortex [6,17], it is likely that this may be one region of the brain important in learning the CWM. We have unpublished data demonstrating differences in learning ability in the CWM and MWM under complete darkness. For example, animals can learn the CWM in complete darkness and they become efficient at solving the task as suggested by the lack of deterioration in performance when the animals are placed in the maze in a lighted room. In the MWM, animals in complete darkness only learn a strategy to locate the platform, but do not show learning beyond this strategy. Furthermore, these animals are initially impaired in performance when placed in the Morris maze under lighted conditions (unpublished observation). Taken

together with previous data showing differences in CWM and MWM performance after various drug administrations [31,38,41,42], the data suggest that the neuroanatomical correlates for learning these mazes are indeed different. Furthermore, this difference in maze performance is not related to some difference in the difficulty of the task, since impairment can occur in either the MWM or the CWM, and therefore appears to be specific to the type of learning (i.e. spatial or sequential).

Interestingly, administration of MA to animals during the same P11–20 time frame as in this study produces only spatial learning and memory deficits but not CWM deficits [38,42]. In this study using MDMA and in a previous study using fenfluramine administration from P11 to 20 [25] we demonstrated that both CWM and MWM learning are impaired. These data suggest that drugs that are potent releasers and reuptake blockers of 5-HT produce a general impairment in learning and memory, whereas a drug such as MA that has a smaller effect on 5-HT produces a more specific learning and memory deficit.

Why only males were affected in the CWM in the present study is unknown and unexpected especially since our previous study with MDMA administration demonstrated that the females showed a somewhat greater impairment in the CWM relative to the males [5]. It is possible that the differences in the experimental design between these studies may account for these changes, although this would have to be investigated further. Coincidentally, this gender difference appeared to be reversed in the MWM during the shifted platform phase, since females that received MDMA had greater difficulties solving the MWM task. It should be noted, however, that an overall main effect of treatment was still observed for this test. Because the males, regardless of treatment, were better than the females at the MWM task, and as suggested above, appear to use a different strategy for solving the maze, the differences in males and females during the shifted platform phase may be related to the type of strategy used. Nonetheless, the spatial deficits were still demonstrated by the males when they were required to learn with a smaller platform that required greater spatial navigation accuracy. These data suggest that over-training with a larger platform may be more beneficial for males than for females, however, when the demands of the task are increased the benefits diminish.

The present data demonstrate that MDMA exposure during a period of brain development that is correlated with third trimester human hippocampal development produces lasting effects on spatial and sequential learning and memory. These changes are not influenced by the reductions in body weight produced by MDMA, the multiple injection procedure used to deliver MDMA, or the litter design used to investigate these effects. Although a mechanism for these effects remains to be elucidated, changes in hippocampal development of 5-HT pathways and/or 5-HT/glucocorticoid interactions appear worthy of further consideration.

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