Pharmacology Biochemistry and Behavior, Vol. 64, No. 4, pp. 705–715, 1999

0091-3057/99/$–see front matter

PII S0091-3057(99)00150-1

The Maternal Separation Paradigm and Adult Emotionality and Cognition in Male and Female Wistar Rats

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LEHMANN, J., C. R. PRYCE, D. BETTSCHEN AND J. FELDON. *The maternal separation paradigm and adult emotionality and cognition in male and female Wistar rats.* PHARMACOL BIOCHEM BEHAV **64**(4) 705–715, 1999.—A single 24-h maternal separation (MS) in the rat during the stress hyporesponsive period alters adult behavior and neuroendocrine stress response. The age of the animal at MS might be a crucial factor for effects in adulthood. We report here on adult behavioral effects of MS performed on postnatal day 4 (MS4), 9 (MS9), or 18 (MS18) in male and female Wistar rats. Unrelated subjects were used to avoid confounding litter effects. Subjects were tested on paradigms of unconditioned fear/anxiety, i.e., open field and elevated plus-maze, and on paradigms involving learning in an aversive situation, i.e., conditioned freezing, active avoidance, and water maze. In line with our predictions we obtained (a) sex differences that were consistent with enhanced fear/anxiety in males relative to females, (b) evidence that MS4 yielded deficits in active avoidance learning and conditioned freezing (trend level), whereas MS9 yielded enhanced active avoidance and water maze learning, (c) evidence (at trend level) that these effects of MS are greater in males than in females. There was no evidence for an effect of MS on paradigms of unconditioned fear/anxiety. We conclude that MS, irrespective of the age at separation, does not provide a robust environmental model of modified behavior in aversive situations. © 1999 Elsevier Science Inc.

Maternal separation Sex differences Open field Elevated plus-maze Conditioned freezing Active avoidance Water maze Rat

ONE approach to the identification of potential animal models of psychiatric disorders involves manipulation of the environment of the individual during its postnatal development followed in adulthood by behavioral and neurobiological screening for induced changes (7,10). In cases where such environmental models include male- or female-specific deficits that are in the same direction as gender-specific symptomology in humans, this represents a real advantage in terms of the opportunity provided to further understanding of the neurobiological development and mechanisms of the disease. In the case of the rat, any attempt to define sex differences in susceptibility to early environmental manipulation needs to take into account the sex differences in emotionality and learning that exist in this species independently of these manipulations (12). In addition to consistent sex differences in nonmanipulated rats with males generally more fearful than females, there is evidence for enhanced male susceptibility to postnatal environmental manipulations (5,14,27,50–52).

Maternal separation is the collective term used to describe a variety of experimental manipulations, all of which involve removal of pups from the dam for time periods of at least 1 h, followed by screening of the effects of the manipulation at specific time points between the manipulation and adulthood. One form of maternal separation (MS) constitutes removal of the litter from the mother for a single and continuous period of 24 h on one specific postnatal day (PND) between birth and weaning. This paradigm has been reported to lead in adulthood to increased basal ACTH and CORT levels [MS on PND 3 (34)], disruption of sensorimotor gating [MS on PND 3, 6, and 9 (8), but see also (18)] as measured with the prepulse inhibition paradigm (16), and disruption of selective attention as measured with the latent inhibition paradigm [MS on PND 10 (6)]. Furthermore, based on these reported long-term behavioral effects, this MS paradigm has been suggested to provide an animal model of symptoms in schizophrenia (7,8). In terms of sex differences, studies based on other environmental models such as early handling and prenatal stress have revealed male–female differences in susceptibility; that is, it has been reported that males are more vulnerable than females (9,27,49,50). In terms of MS, to the best

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of our knowledge, only sensorimotor gating has been studied for a differential sex effect; no male–female difference was reported (8,18).

One variable that is potentially important in terms of environmental models is the age/stage of development of the rat pup on exposure to the manipulation [e.g., (7)]. In this respect, one major advantage of the single MS paradigm relative to repeated separation paradigms is the opportunity it provides to investigate the specificity of the manipulation in terms of the pup's stage of development. In rat pups PND 4–14 are characterized by low basal HPA activity and HPA resistance to typical stressors; accordingly, this age has been designated as the stress hyporesponsive period (SHRP) of rat development (19,36,48). MS for 24 h at any age during the SHRP yields a stress response in the immediate term [e.g., (20)]. Thus, when the effects of MS are measured immediately after the 24 h, the pups, irrespective of age at MS (PND 3–20), display increased "basal" ACTH or corticosterone (CORT) levels (2,17,20,28,41) and an increased CORT response to mild stressors such as novelty or saline injection (20,38,40,41). Therefore, whether or not the age of the pup at MS is an important factor depends on what parameter is being measured and the developmental stage of the subject at test. When assessed at weaning age (PND 20), rats separated at PND 3, 7, or 11 do not display any change in basal corticosterone or ACTH, but in terms of stressor responsiveness (to saline injection), MS at PND 3 results in an increase in pituitary–adrenal activity (ACTH release) relative to nonseparated controls, while rats separated at PND 7 or PND 11 display a decrease in pituitary–adrenal activity at weaning (44,45). Although there are no differences between males and females in this latter respect, van Oers et al. (44) do report sex differences in response to MS in terms of expression of mineralocorticoid receptor (MR) mRNA in the paraventricular nucleus (PVN) of the hypothalamus at PND 20: whereas males exposed to MS on PND 3 show an increase, females do not show any difference compared to control subjects; and on the other hand, females display a decrease in MR mRNA following MS on PND 11, which does not seem to affect males at this age [see also (42)].

Here we report a large-scale investigation into the effects of MS on unconditioned (novelty) fear and aversive conditioning in adulthood, in which the independent variables of interest were age at MS relative to the SHRP—PND 4 vs. 9 vs. 18—and sex. Given that MS early in the SHRP leads to stress hyperresponsiveness, whereas MS later in the SHRP leads to stress hyporesponsiveness, we predicted that MS carried out at the beginning (PND 4) or in the middle (PND 9) of the quiescent period of HPA axis development would lead to impairment and improvement, respectively, of performance in behavioral paradigms with an aversive component, whereas MS at PND 18 (after the SHRP) would have no effect. With regard to sex, we predicted that the effects at PND 4 and PND 9 would be more pronounced in male offspring, given the evidence for relative male susceptibility to behavioral disturbance by the postnatal environment (9,27,49,50). The paradigms on which the effects of MS were analyzed and compared in terms of age and sex were open field, elevated plus-maze, aversive conditioning to context, and a discrete stimulus measured as freezing, active avoidance, and water maze. These paradigms, some of which have already been used to screen for effects of environmental manipulations (22,23,43,51), provided a comprehensive screening battery for MS-induced effects of unconditioned fear/anxiety and on behavioral conditioning in an aversive situation.

METHOD

Subjects

The experiments were carried out with male and female Wistar rats, all bred in house [Zur:WIST(HanIbm) Animal Services, Swiss Federal Institute of Technology Zürich, Schwerzenbach], and maintained under constant husbandry conditions of reversed cycle lighting (lights on: 1900–0700 h) in a temperature (21 ± 1°C)- and humidity (55 ± 5%)-controlled animal facility. All the experiments were carried out in accordance with the Swiss Federal Regulations for animal experimentation. Subjects were derived from 48 different litters. All litters were born within a range of 5 days. Within 24 h after birth all litters from which subjects were derived were culled to the same litter size and composition of four males and four females. To minimize disturbance to litters that might confound effects of the MS procedure (see below), cage cleaning was carried out once only, at PND 11, for all litters between birth and weaning (day 21). At weaning subjects were weighed and placed in group cages (Perspex Macrolon type IV, 59.0 × 38.5 × 20.0 cm), four same-sex animals per cage, with each group of four animals derived from different litters and belonging to the same treatment group. Subjects remained undisturbed until testing started at age 3 months, with food (Kliba 3430, Klibamühlen, Kaiseraugst, CH) and water available ad lib. For testing, subjects were divided into three independent groups: 64 animals (8 subjects per sex and treatment group) for the open field, 96 animals in the freezing paradigm (12 subjects per sex and group), and 64 animals tested sequentially on plus maze, then 2 days later in active avoidance and, finally, 3 months later in the water maze (8 subjects per sex and group). Within group and sex, subjects were derived from different litters, i.e., they were unrelated.

Maternal Separation Procedure

Three experimental groups and one control group were studied. MS subjects were separated from their mothers for 24 h, 1800–1800 h, at PND 4 (MS4), or day 9 (MS9), or day 18 (MS18). To carry out MS, the dam was removed to another cage while the pups remained in their home cage. The home cage was then transferred to a separate room and placed on a heat pad set at 33°C. After 24 h the pups were returned to the colony room and the mother returned to the home cage. Control animals (CON), apart from culling and the single cage cleaning, remained undisturbed with their mothers from birth until weaning.

Weaning Weights

At weaning on PND 21 all pups were weighed. Weights were analyzed for MS and sex differences with a nested 4 × 2 ANOVA with the factors of MS (CON, MS4, MS9, MS18) and sex (male, female), and litters nested within MS as the appropriate error term for the MS effect. This analysis accounts for the fact that pups derived from the same litter cannot be treated as independent data points.

EXPERIMENTS

Spontaneous Locomotor Activity in an Open Field

Apparatus. Locomotor activity was measured in four open-field (76.5 × 76.5 × 49 cm) arenas made of dark gray plastic. Behavior in the arenas was recorded by a video camera mounted on the ceiling and relayed to a monitor and a video

tracking, motion-analysis and behavior-recognition system (EthoVision, Noldus, Wageningen, The Netherlands). The test room was dimly illuminated with indirect lighting (three 60-W bulbs).

Animals and procedure. The experiment was performed on a total of 64 3-month-old naive rats without any pretest handling in adulthood. Rats were run in squads of four, and all animals were habituated to the testing room for 20 min before the start of each session. Spontaneous locomotion and habituation were assessed in five consecutive daily sessions. Animals were tested at the same time each day, and each rat was placed in the center of one of four arenas and allowed to explore it for 30 min.

Data collection and analysis. The computer software (EthoVision) calculated the total distance a rat traveled while in the arena. All data were analyzed by a $2 \times 4 \times 5$ ANOVA, with the main factors sex (male, female), MS (MS4, MS9, MS18, CON), and the repeated-measures factor of days (1 to 5). In addition, the data of day 1 of testing were analyzed separately by a $2 \times 4 \times 6$ ANOVA, with the main factors sex (male, female) and MS (MS4, MS9, MS18, CON), and a repeated-measures factor of bins (six bins of 5 min).

Conditioned Freezing

Apparatus. The freezing apparatus and method of data collection have been described in detail elsewhere (31). Briefly, four identical Coulbourn Habitest operant test chambers were used for assessment of contextual and discrete stimulus conditioning (model no. E10-10RF). These had two aluminium walls and two Perspex walls. The house light remained off throughout the experimental sessions in this chamber. The chamber floor consisted of 16 stainless steel bars with a diameter of 5 mm, spaced 1.3 cm apart, through which shocks could be delivered. Presentation of stimuli was controlled by a Coulbourn Universal Environment Interface (model no. E91-12) and a Coulbourn Universal Environmental Port (model no. L91-12). Shocks were delivered, where appropriate, via a Coulbourn Precision Regulated Animal Shocker (model no. E13-12). Attached to the center of each chamber's ceiling was a minivideo camera equipped with a wide-angle (100°) 2.5-mm lens. Data analysis was carried out on line using a computer-controlled script derived from the "NIH IMAGE" program (available from web-site http://rsb.info.nih.gov/nih-image). Our script worked by comparing every 2 adjacent s of video tape to generate a screen representing the percentage difference between them. This percentage was used as an index of locomotor activity. We classified a difference of less than 0.05% (50 pixels) as a freezing response.

Animals and procedure. The experiment was performed on 96 4-month-old rats, run in squads of four, with boxes counterbalanced across MS groups. Rats were placed in the conditioning chamber for 30 min. Each received 10 pairings of an 85dB[A] tone (conditioned stimulus, CS) and foot shock. For all rats the tone had a duration of 30 s. The shock had a duration of 1 s and intensity of 0.4 mA. Tone offset was timed to coincide precisely with foot shock onset. The intertrial interval (ITI) was 2 min. For all rats the first trial began 150 s after the start of the session. Twenty-four hours later, rats were placed in the conditioning chamber for 8 min and conditioned freezing to contextual cues was assessed. Forty-eight hours after conditioning, all rats were placed again in the same chamber for 11 min; after 3 min the tone was presented continuously for 8 min. Freezing during the pretone period as well as during the tone presentation was measured.

Data analysis. Conditioning data were analyzed by a $2 \times 4 \times 10$ ANOVA with main factors of sex, MS, and a repeated measures factor of CS presentation (10) to analyze the freezing response during CS exposure. A separate $2 \times 4 \times 11$ ANOVA with main factors sex, MS, and a repeated-measure factor of ITIs (11) was performed. Conditioned freezing to the contextual cues and to the tone during the test sessions was analyzed using two separate $2 \times 4 \times 8$ ANOVAs and one $2 \times 4 \times 3$ ANOVA with main factors of sex, MS, and a repeated-measurements factor of bins (pre-CS freezing: 3×1 min; context and CS tests: 8×1 min).

Elevated Plus-Maze

Apparatus. The maze was constructed of black painted wood. It was arranged as a cross, with two open arms facing each other. The other two arms were enclosed by 40-cm high walls. The arms measured 45×10 cm, and were raised by a single central support to a height of 62 cm above the floor. The four arms extended from a common central platform (10×10 cm). Exploration of the open arms was encouraged by testing under dim light (2×60 W indirect). Behavior on the maze was recorded via a video camera mounted on the ceiling above the center of the maze and relayed to a monitor and a video tracking, motion-analysis and behavior-recognition system (EthoVision®, Noldus, Wageningen, The Netherlands). The maze was divided into five areas: one for each arm and one for the center. Equipment programming and data recording were controlled by a Compaq IBM-compatible personal computer (486/DX2/66).

Animals and procedure. A total of 64 naive adult 5-month-old rats were tested individually on the plus-maze without any pretest handling in adulthood. Each rat was habituated to the testing room for at least 20 min before being placed on the central platform facing one of the open arms and allowed to explore the maze for 5 min, after which it was removed from the maze and returned to its home cage. The computer software calculated the time the animal spent in each part of the maze, the number of entries into open and closed arms and the total distance the rat traveled while in the maze.

Data analysis. The number of entries and time spent in the open arms and total distance traveled was analyzed by three separate 2×4 ANOVAs with main factors of sex and MS.

Active Avoidance

Apparatus. The apparatus consisted of four identical Coulbourn Instruments shuttle boxes (Model E10-16TC), each set in a ventilated, sound and light-attenuating shell (Model E10-20). The internal dimensions of each chamber were $35 \times 17 \times 21.5$ cm, as measured from the raised grid floor. The box was divided by an aluminium hurdle (17 cm long, 4 cm high). The barrier was very thin to prevent animals from balancing on it and thus avoiding shock. Scrambled shocks were delivered from a constant current shock generator (CI, Model E13-14) and scanner (Model E13-13) set at 0.5 mA. The chambers were illuminated during the experimental session with two diffuse light sources (house lights), mounted 19 cm above the grid floor in the middle of the side walls. The conditioned stimulus (CS) was an 85-dB[A] tone (10 s) produced by a 2.9-kHz tone module (Model E12-02) placed behind the shuttle box on the floor of the shell.

Animals and procedure. For the two-way active avoidance paradigm a total of 64 5-month-old rats were tested. One-half of these were preexposed to the tone that we used as the conditioned stimulus, as part of a study of the effects of MS on la-

tent inhibition. Only the procedure and data for the nonpreexposed subjects ($n = 32$) are presented here. Rats were run in squads of four. The procedure included two stages given 24 h apart.

Exposure to the apparatus: each animal was placed in the shuttle box with the house light on for a period of 60 min on 2 consecutive days.

Test: each animal was placed into the shuttle box and received 80 avoidance trials on a variable interval schedule of 50 s, ranging from 10 to 90 s. Each avoidance trial began with a 10-s tone followed by 2 s of 0.5 mA shock, the tone remaining on with the shock. If the animal crossed the barrier to the opposite compartment during the tone, the stimulus was terminated and no shock was delivered (avoidance response). A crossing response during shock terminated the tone and the shock (escape response). If the animal failed to cross during the entire tone–shock trial, the tone and the shock terminated after 12 s. The number of avoidance responses was recorded in blocks of 10 trials.

Data collection and analysis. The 80 avoidance trials of the test session were divided into eight blocks of 10 trials each. The number of avoidance responses per 10 trials was calculated for each of the eight blocks. These data were analyzed by $2 \times 4 \times 8$ ANOVA, with main factors of sex (male, female), MS (MS4, MS9, MS18, CON), and a repeated-measurement factor of blocks (1–8) each consisting of ten trials.

Water Maze

Apparatus. The Morris water maze consisted of a circular black pool (2.0-m diameter) filled with tap water (maintained at 21 ± 1°C; water level 30 cm below the rim of the pool), and situated in a room (3.4 m × 4.9 cm × 2.9 m) with a variety of visual extramaze cues. Depending on the protocol, the pool contained a hidden circular platform (11 cm diameter, 2 cm below water surface, painted black) during acquisition and reversal training, a "visible" platform (hidden platform with a white disk of the same diameter 10 cm above the water surface) during cue training, or no platform during the probe trial. The rough surface of the platform made it easy for the animals to climb onto it. Four equally spaced points at the border of the pool were designated as N, E, S, W, and used as starting points for swim trials.

Animals and procedure. The experiment was performed on a total of 64 9-month-old rats. During acquisition training (days 1–6) animals were trained with the hidden platform in a constant position 43 cm away from the pool wall. Four trials per day were given and the starting points varied in a random order. Trials started with the rat facing the wall of the pool. Rats that did not find the platform within 60 s were guided to the platform. Further, after climbing onto the platform animals were left there for 20 s. The trial was terminated by removing the rat from the water tank and either placing it back into the tank for another trial at a different starting point or—after the completion of a trial block—returning it to its home cage after drying it with a towel.

For the probe trial (day 7), the platform was removed and each animal was allowed to search for the platform for 60 s (one trial only). On the day following the probe trial, reversal training similar to the acquisition training started. Animals were trained to find the hidden platform, which had now been moved to a position diagonally across from the initial location, for 4 days (days 8–11). On day 12, all animals were submitted to a second probe trial. Finally, on day 13, all subjects were tested for their ability to locate and climb onto the visible escape platform. Each animal was submitted to a block of four consecutive trials, beginning at a constant starting point. The rat was then allowed to locate and climb onto the visible platform, which was placed at varying positions in the pool. All animals were allowed to spend 20 s sitting on the platform.

Data collection and analysis. The following data were recorded per trial and analyzed using a computer based video tracking system (HVS Image, UK): escape latency (i.e., the time it took the animal to find the platform and climb onto it), escape distance (i.e., distance swum during the escape latency), and speed. For the probe trial, the percentage of total time spent and distance swum in each quadrant of the pool were determined automatically. Data were analyzed by a $2 \times 4 \times 6/4$ ANOVA with the main factors of sex (male, female), MS (MS4, MS9, MS18, CON), and a repeated-measurement factor of days (6)/(4) days for acquisition/reversal training, respectively. Speed data were averaged over days and analyzed by a 2×4 ANOVA, with the main factors of sex and MS. Probe data were analyzed by a $2 \times 4 \times 4$ ANOVA, with the main factors of sex (male, female) and MS (MS4, MS9, MS18, CON), and a repeated-measurement factor of quadrants (4). Because the fourth quadrant data point is never independent of the other three, the p-values were adjusted to reflect a reduction in degrees of freedom in the effect of quadrant and the quadrant interactions. Data of the visible platform test were analyzed using a 2×4 ANOVA, with the main factors of sex (male, female) and MS (MS4, MS9, MS18, CON).

RESULTS

Weaning Weights

Mean values of body weights at weaning are given in Table 1. The "nested design" ANOVA revealed: a significant effect for litter within MS, $F(44, 332) = 21.45$, $p < 0.001$, indicating weight differences between litters within the treatment groups; a significant sex effect, $F(1, 332) = 43.8$, $p < 0.001$, with males heavier than females; a significant effect of MS, $F(3, 44) = 9.19$, $p < 0.001$, demonstrating that all MS groups were lighter than control subjects. In addition, there was a significant interaction of sex × MS, $F(3, 332) = 3.25$, $p < 0.03$, indicating that while there was a significant sex difference in CON, MS4, and MS18 subjects, no such difference was present in MS9 subjects. These differences in body weight were not present in subjects at 3 months of age. At this age only the factor sex revealed a significant effect, $F(1, 56) = 476.1$, $p < 0.001$, with males being heavier than females (males: 327.28 ± 4.97, females: 202.25 ± 2.8).

Spontaneous Locomotor Activity in an Open Field

In terms of total distance moved, the $2 \times 4 \times 5$ ANOVA revealed a strong tendency for a main effect of sex, $F(1, 56) = 3.3$, $p < 0.08$, demonstrating a trend towards the typical situa-

TABLE 1

WEANING WEIGHTS (MEANS ± SEM)

	Female	Male
CON	47.75 ± 0.68	50.16 ± 0.75
MS4	42.71 ± 0.42	44.80 ± 0.38
MS9	41.60 ± 0.60	42.36 ± 0.57
MS18	42.95 ± 0.45	44.06 ± 0.54

tion that females are more active than males (as can be seen in Fig. 1, a sex difference did not exist in the MS18 group). Neither a significant main effect ($p > 0.63$) nor interaction ($p > 0.24$) involving MS was found. There was a significant effect of days, $F(4, 224) = 8.1$, $p < 0.001$, reflecting habituation over the 5 days of testing (see inset in Fig. 1), but no interaction between days and either sex or MS. A separate analysis of the first day of testing revealed a significant sex × bins interaction, $F(5, 280) = 3.73$, $p < 0.03$, indicating higher activity during the first 5 min of open-field testing in females than in males, with subsequent habituation in both sexes.

Conditioned Freezing

CS conditioning. The 2 × 4 × 10 ANOVA revealed no significant main effect or interaction involving the factor of MS ($p > 0.56$, interaction: $p > 0.91$). The repeated-measurements factor of CS presentation revealed a significant effect, $F(9, 792) = 54.4$, $p < 0.001$, reflecting an overall increase in freezing following the first two presentations of the CS and thereafter a stable asymptote of about 50% (see Fig. 2a). In addition, the interaction of sex × CS presentation was significant, $F(9, 792) = 2.6$, $p < 0.006$, reflecting increased freezing in males compared to females following the first conditioning trial, i.e., during the second CS presentation only. Thereafter, both sexes displayed a comparable level of freezing (see Fig. 2a).

ITI conditioning. The 2 × 4 × 11 ANOVA revealed no significant main effects or interactions involving the factors sex ($p > 0.39$) or MS ($p > 0.79$; interaction: $p > 0.50$). Only the repeated-measures factor of ITIs yielded a significant effect, $F(10, 880) = 45.1$, $p < 0.001$, demonstrating an increase in freezing following the first two CS-US pairings up to a level of 50% and thereafter during the last six ITIs a stable freezing level of around 35%, which is depicted in Fig. 2b.

Context test. The 2 × 4 × 8 ANOVA did not yield a main effect of sex ($p > 0.36$) or MS ($p > 0.24$). However, their interaction approached significance ($p < 0.10$), reflecting that although in females the overall level of freezing to context was similar in controls and the three MS groups, in males overall freezing was highest in the CON group (which was also higher than CON females) and reduced in the MS groups (MS9 and MS18). Male subjects separated on PND 18 showed the lowest level of freezing, also when compared to their female counterparts, as depicted in Fig. 2c. In addition the overall analysis revealed a significant effect of bins, $F(7, 616) = 7.3$, $p < 0.001$, reflecting an increase in freezing during the first 3 min to a level of 30% and a gradual decrease during the following 5 min to a level of 15% (see inset histogram). However, no significant interactions of bins with either sex ($p > 0.79$) or MS ($p > 0.36$) were found.

Tone test. The 2 × 4 × 3 ANOVA of pretone freezing response did not yield either a main effect or interaction involving the factor sex or MS. There was a significant effect of bins, $F(2, 176) = 10.21$, $p < 0.001$, reflecting an increase in freezing during the first 3 min to a level of 5%. The 2 × 4 × 8 ANOVA of freezing in response to the tone did not yield either a main effect or an interaction involving the factors sex ($p > 0.84$) or MS ($p > 0.89$). There was a significant effect of bins, $F(7, 616) = 47.6$, $p < 0.001$, reflecting an increase in freezing during the first 3 min to a level of 60% and a gradual decrease during the following 5 min to a level of 20%. In addition, the interaction of bins × MS approached significance

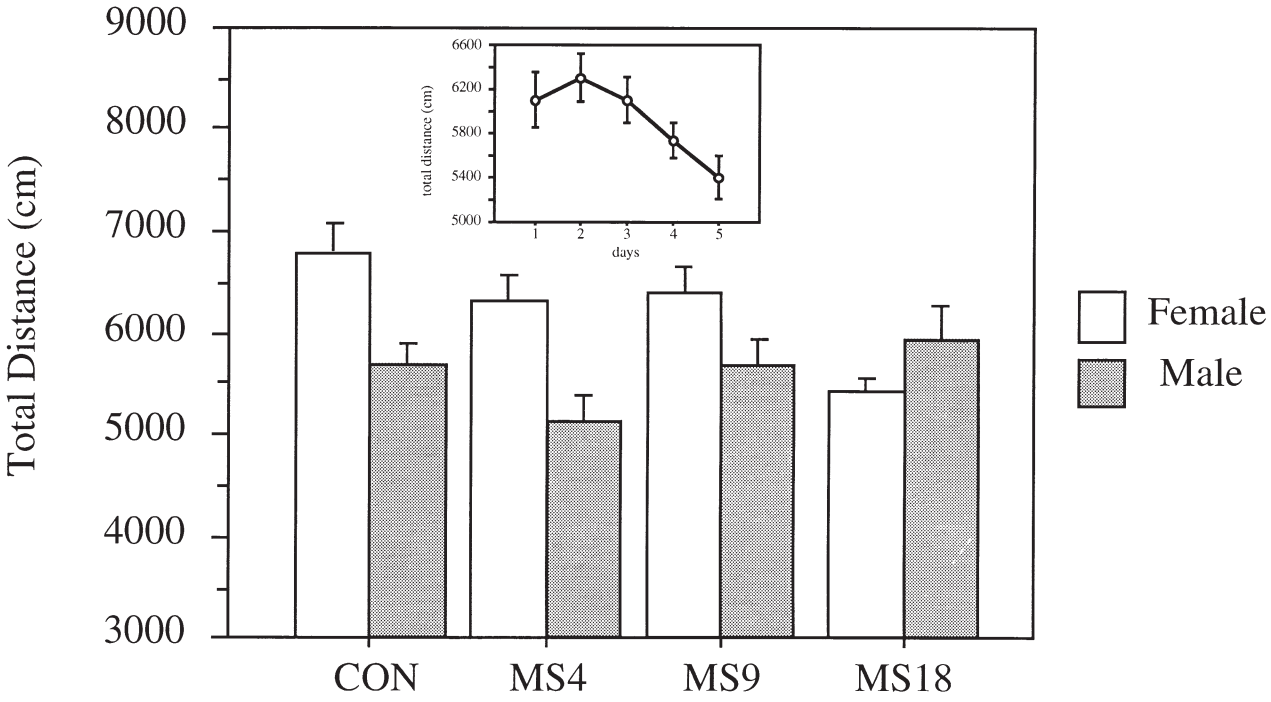

FIG. 1. Total distance moved in an open-field arena by adult male and female rats following maternal separation on PND 4 (MS4), 9 (MS9), or 18 (MS18) or following uninterrupted maternal care (CON). The bars represent means ± SEM over 5 days of testing ($n = 8$ animals per group). The inset histogram depicts overall attenuation of total distance moved across the 5 days of testing.

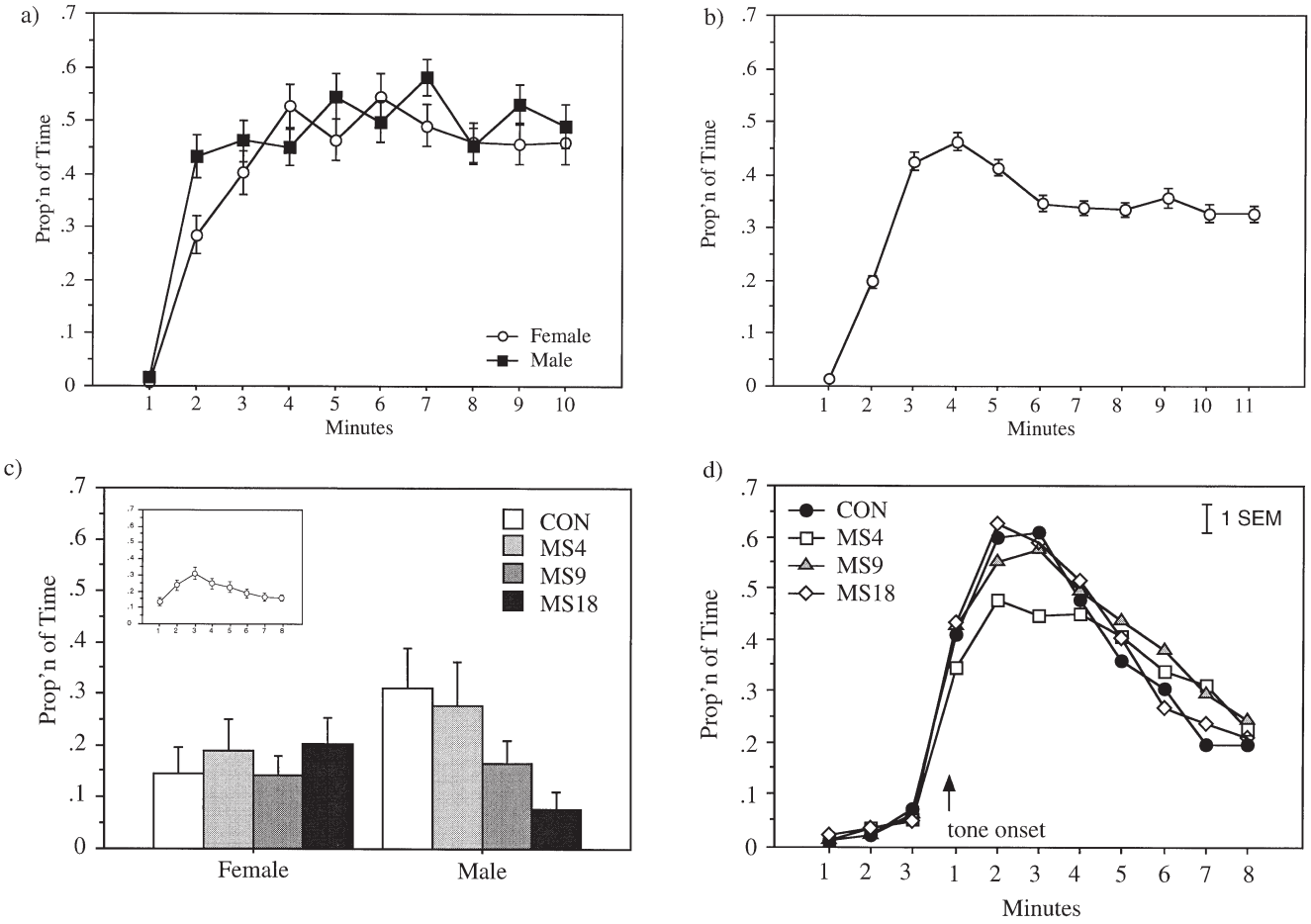

FIG. 2. Conditioned freezing. (a) Acquisition. Proportion of time spent freezing during ten 30-s CS presentations. Overall means ± SEMs for male ($n = 48$) and female ($n = 48$) rats. (b) Acquisition. Proportion of time spent freezing during 11 2-min intertrial intervals. Overall means ± SEMs for all subjects combined ($n = 96$). (c) Expression of context conditioning. Proportion of time spent freezing in response to contextual cues by male and female subjects with or without maternal separation (abbreviations as in Fig. 1). The bars represent mean values ± SEM over 8 min of testing ($n = 12$ animals per group). Inset histogram displays the time course of the freezing response during the 8 min of testing for all subjects combined. (d) Expression of CS conditioning. Proportion of time spent freezing across three 1-min bins pre-CS and eight 1-min bins of CS (tone) presentation in subjects with or without maternal separation; males and females combined ($n = 24$ per treatment group).

($p < 0.08$), reflecting a lower peak in freezing response in subjects separated on PND 4 compared to the other groups, which were very similar to each other (Fig. 2d). It is interesting to note that the maximal level of freezing in the tone test was higher than during the context test: 60% compared to 30%.

Elevated Plus-Maze Activity

The 2 × 4 ANOVA of number of entries into the open arms and of time spent in the open arms did not yield either a main effect or an interaction involving the factors sex ($p > 0.99$, $p > 0.11$) or MS ($p > 0.83$, $p > 0.30$, interaction: $p > 0.33$, $p > 0.13$). However, there was a significant effect of sex on total distance traveled, $F(1, 56) = 5.7$, $p < 0.02$, demonstrating that females were more active than males, irrespective of MS (females: 706.06 ± 28.89, males: 617.67 ± 22.87). MS did not yield a significant main effect or interaction ($p > 0.24$, interaction: $p > 0.74$).

Active Avoidance Paradigm

The 2 × 4 × 8 ANOVA of avoidance responses yielded an almost significant main effect of MS, $F(3, 24) = 2.99$, $p < 0.06$, a significant main effect of sex, $F(1, 24) = 10.74$, $p < 0.004$, and a significant interaction of MS × sex, $F(3, 24) = 4.06$, $p < 0.02$. In addition, there was a significant effect of blocks, $F(7, 168) = 28.41$, $p < 0.001$, indicating that the number of avoidance responses increased over blocks: gradual acquisition of the avoidance response. As can be seen in Fig. 3, the significant interaction of sex × MS reflects that although there was no significant difference between treatment groups in females, within males, subjects separated on PND 4 displayed a severe deficit in avoidance learning, i.e., the number of avoidance responses was much lower in MS4 males compared to all other groups. This interpretation was supported by 2 post hoc 4 × 8 ANOVAs for males and females separately, yielding a significant main effect of MS, $F(3, 12) = 6.18$, $p < 0.01$, and interaction of MS × blocks, $F(21, 84) = 2.92$, $p < 0.001$, in males but not in females ($p > 0.42$, $p > 0.88$). As can also be

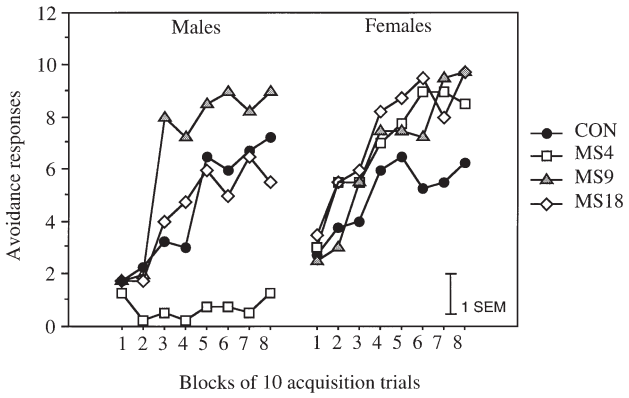

FIG. 3. Number of avoidance responses in the active avoidance paradigm, expressed as mean values ($n = 4$ per group) over eight blocks of 10 trials, in males and females. Maternal separation abbreviations as in Fig. 1.

seen in Fig. 3, MS9 males displayed accelerated avoidance learning in comparison with CON subjects, supported by a significant interaction of MS and blocks in a separate post hoc analysis comparing CON and MS9 male subjects, $F(7, 42) = 2.3$, $p < 0.05$.

Water Maze: Acquisition

Latency to reach platform. The $2 \times 4 \times 6$ ANOVA did not yield a significant main effect or interaction of sex ($p > 0.32$) or MS ($p > 0.50$, interaction: $p > 0.52$), but revealed a significant effect of days, $F(5, 280) = 42.9$, $p < 0.001$, confirming a reduction in latency to escape over days. The interaction days $\times$ sex approached significance ($p < 0.07$), reflecting that while there was no sex difference on day 1, males tended to display shorter latencies to reach the platform than females during subsequent days (Fig. 4a). There were no interactions of days $\times$ MS ($p > 0.97$) or days $\times$ sex $\times$ MS ($p > 0.19$). As depicted in Fig. 4b, and as confirmed by a separate analysis, there was accelerated learning in MS9 relative to CON males ($p < 0.04$).

Distance to reach platform. The $2 \times 4 \times 6$ ANOVA yielded a significant effect of sex, $F(1, 56) = 6.8$, $p < 0.02$, reflecting shorter distances swum by males than by females, and a significant effect of day, $F(5, 280) = 37.5$, $p < 0.001$, reflecting a decrease in distance to find the platform. MS did not affect distance to reach the platform, neither as a main effect ($p > 0.31$) nor as an interaction ($p > 0.96$).

Speed. The 2×4 ANOVA yielded a main effect of sex, $F(1, 56) = 5.5$, $p < 0.03$, reflecting that overall females (25.98 $\pm$ 0.18 cm/s) swam faster than males (24.49 $\pm$ 0.21 cm/s).

Probe Trial 1

The $2 \times 4 \times 4$ ANOVA yielded only a significant effect of quadrant, $F(2, 168) = 15.51$, $p < 0.001$, indicating that overall the animals spent more time in the quadrant where the platform was situated during acquisition training than in the other quadrants.

Reversal

The $2 \times 4 \times 4$ ANOVA yielded a significant effect of sex, $F(1, 56) = 8.6$, $p < 0.005$, showing that males acquired the task much faster and to a greater extent than females. MS did not reveal a significant main effect ($p > 0.64$) or interaction

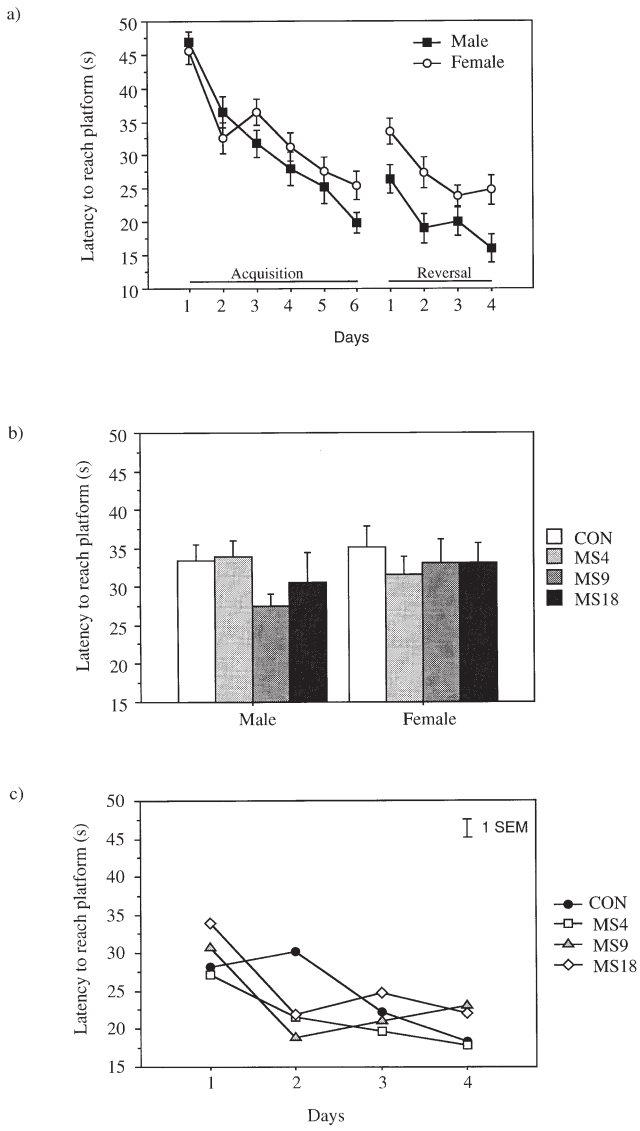

FIG. 4. Water maze. (a) Means ($\pm$SEM) of latencies (seconds) to reach platform for male ($n = 32$) and female ($n = 32$) rats (all treatments combined) across 6 days of acquisition and 4 days of reversal training. (b) Average latency (seconds) to reach platform for male and female rats according to maternal separation treatment. The bars represent mean values $\pm$ SEMs collapsed over 6 days of acquisition training ($n = 8$ animals per group). (c) Latency to reach platform during reversal training, according to maternal separation treatment with males and females combined ($n = 16$ animals per group).

with sex ($p > 0.78$). There was a significant effect of days, $F(3, 168) = 16.7$, $p < 0.001$, demonstrating the overall learning across the 4 days of reversal. Additionally, the day $\times$ MS interaction was significant, $F(9, 168) = 2.5$, $p < 0.02$, reflecting that all MS groups improved significantly on day 2 of training, while the CON subjects reached a comparable level of performance only after 3 days of training (Fig. 4c). This finding was irrespective of sex ($p > 0.33$). With regard to distance, the $2 \times 4 \times 4$ ANOVA yielded the same outcomes as the latency, and with regard to speed, the 2×4 ANOVA did not yield either a main effect or an interaction involving the factors sex ($p > 0.23$) and MS ($p > 0.34$, interaction: $p > 0.81$).

Probe Trial 2

The 2 × 4 × 4 ANOVA yielded a significant effect of quadrant, $F(2, 168) = 92.34$, $p < 0.001$, indicating that all subjects spent more time in the quadrant that contained the platform during the reversal training.

Visual Test

The 2 × 4 ANOVA for the latency to reach the platform yielded no significant effect or interaction of MS or sex.

Table 2 provides a summary of the significant main effects and interactions, or trends thereof, of MS and sex, as reported in the results above.

DISCUSSION

The present study aimed to assess long-term consequences of a 24-h MS on emotionality and cognition as a function of the age of pups relative to the SHRP at time of separation and as a function of sex. The major findings of this study were as follows: 1) a sex difference (at least at trend level) was obtained on the majority of paradigms, and these sex differences were in accordance with those well documented in the literature; 2) as predicted, MS4 subjects tended to demonstrate impaired performance and MS9 subjects improved performance, relative to CON subjects, in aversive learning, but contrary to prediction there was no effect of MS on fear of novelty as measured by open field activity and plus maze; 3) as predicted, MS18 was the least effective manipulation; 4) as predicted, males tended to be more affected by MS than females.

In terms of sex differences per se, our findings are consistent across paradigms and consistent with those reported in the literature: increased female activity as measured on the elevated plus maze and in the open field (3,13,14,30), faster female acquisition in active avoidance (35), faster rate of development of conditioned freezing in males (21,29), and superior performance of males in the water maze (11,33). This high degree of consistency represents a robust validation of the present study and the methodology used. On the basis of this validation we will now proceed to a discussion of the effects of MS.

The physical severity of MS, at least in the relative short term, is indicated by the reduction in body weight at weaning demonstrated by all three MS groups, males and females, relative to controls. In adulthood (age 3 months) no weight differences between CON and MS groups were observed. In terms of an overall behavioral effect, i.e., an effect of MS in adulthood irrespective of age at separation and sex, this was only obtained with water maze reversal learning, which was improved in all MS groups when compared to CON on day 2. However, based on our hypotheses, overall behavioral effects were not expected; rather, based on the evidence for stress hyperresponsiveness following MS conducted early in the SHRP and stress hyporesponsiveness following MS later in the SHRP (44,45), we hypothesized differential effects of MS on PND 4 vs. 9. Although in part only at the trend level, those MS effects that we did observe were consistent with this hypothesis: this was the case in conditioned freezing, active avoidance, and in the water maze. Although MS4 did not affect conditioned freezing to context, it did result in decreased CS conditioning, and consistent with this was the finding that MS4 males failed to acquire active avoidance. The deficit in acquisition of active avoidance by MS4 males represents one of the most notable findings of this study. Interestingly, we have observed this same combination of low expression of CS-conditioned freezing and low acquisition of active avoidance in males of the inbred Lewis strain (29,39). In contrast, MS9 males displayed normal (i.e., not different from CON level) CS conditioning, and a tendency towards enhanced acquisition of active avoidance and enhanced spatial learning in the water maze. These findings are in line with our expectation that MS in the middle of the SHRP (day 7 or later) leads to enhanced behavioral performance in aversive environments. The only existing study of the effect of MS on conditioning is that which describes the absence of an effect of MS at PND 10 in the conditioned taste aversion paradigm (6),

TABLE 2

SUMMARY OF THE SIGNIFICANT AND TREND ($0.05 < p < 0.10$) EFFECTS OF MATERNAL SEPARATION AND SEX

Paradigm	Parameter Measured	Factor	$p <$	Effect/Trend
Body weight	Weight at weaning	Sex	0.001	Males heavier than females
		MS	0.001	All MS groups lighter than CON
		Sex × MS	0.03	No sex difference in MS9
Open field	Activity	Sex	0.08	Females more active than males, but no such sex effect in MS18 masked an overall sex difference
Freezing: conditioning	CS conditioning	Sex × CS	0.006	Males reached asymptote faster than females
Freezing: context	Expression of context conditioning	Sex × MS	0.10	Male-specific reduction in MS9 and MS18
Freezing: tone	Expression of CS conditioning	Bins × MS	0.08	Reduction in MS4
Plus maze	Activity	Sex	0.02	Females more active than males
Active avoidance	Conditioning to CS	MS	0.06	Deficit in MS4 (males)
		Sex	0.004	Females learn faster than males
		Sex × MS	0.02	Deficit in MS4 males, accelerated learning in MS9 males
Water maze	Spatial learning (latency)	Day × Sex	0.07	Superior learning in males from day 2 onwards (within males, superior learning in MS9)
Water maze	Spatial learning (distance)	Sex	0.02	Superior learning in males
Water maze	Speed	Sex	0.03	Females faster than males
Water maze	Rule reversal	Sex	0.005	Superior learning in males
		Day × MS	0.02	Superior learning in all MS groups on day 2

which is somewhat contrary to our findings in MS9 males, but may reflect differences in conditioning paradigms.

Most unexpected was our finding that MS at PND 4, 9, or 18 did not affect performance in either of the two paradigms of spontaneous fear/anxiety. It is known that MS at PND 7 or 11 reduces HPA responsiveness at weaning age, and that MS at PND 3 increases HPA responsiveness at weaning age (44) and in adulthood (34). Based on the evidence that changes in HPA responsiveness correlate highly with changes in emotionality/anxiety (43), we had predicted that MS at PND 9 would yield reduced fear/anxiety in the open field and on the elevated plus maze (15,43), while MS at PND 4 was expected to enhance behavioral expression of fear/anxiety. Neither of these predictions was supported by our data. One conclusion that can be drawn from these negative findings is that effects of a manipulation on the HPA axis (34) cannot necessarily be used to predict effects of the same manipulation on fear/anxiety expression at the behavioral level.

Our hypothesis that MS carried out as late as PND 18 would not affect behavior was largely but not entirely supported. The only deviation was the tendency for an absence of sex differences in MS18 subjects on several paradigms with which we obtained the expected sex differences with CON: this appeared to be the case for locomotor activity in the open field due to reduced activity in females and for context conditioning due to reduced freezing in males. Additionally, and irrespective of sex, MS18 subjects demonstrated superior rule reversal learning in the water maze task. Thus, the observed effects of MS18 could not be attributed to one sex specifically, but rather demonstrated a loss of sex specific behavioral patterns in both males and females, suggesting that also after the SHRP maternal contact remains essential to typical male and female rat development (3,32).

Only tasks involving learning under aversive conditions were or tended to be affected by MS, i.e., conditioned freezing, active avoidance learning, and water maze performance, and, furthermore, on all of these paradigms the MS effect was or tended to be sex specific. In the freezing paradigm, context conditioning was reduced in MS9 and MS18 males, with females remaining unaffected. In the active avoidance paradigm, only males displayed an effect of MS, in the form of a severe deficit and an improvement in MS4 and MS9, respectively. Again, females did not differ significantly between groups. A similar effect was seen in water maze acquisition—while females did not differ between groups, MS9 males displayed enhanced learning. On the other hand, females as well as males exposed to MS displayed weight deficits at weaning, reduced freezing to CS (MS4) and improved reversal in the water maze. This indicates clearly that both males and females are affected by MS, but that in males the effect translates more readily into behavior. However, the finding that males are more susceptible than females to MS refers to long-term effects only, because many studies that analyzed the acute neuroendocrine effects of MS in both male and female rats reported no sex difference, i.e., equivalent susceptibility (20,40,41). There is no doubt that females are affected by some early environmental manipulations (26), as reported by van Oers et al. (44), the present study, and in many studies using other environmental manipulations (e.g., prenatal stress, repeated MS). However, effects in females are often less severe, and can be distinguished from effects in males, behaviorally and neuroanatomically. There are several putative mechanisms that could account for sex specific differences in response to MS. It has been suggested that maternal behavior plays a critical role in HPA axis development, and that the effects of early manipulations are at least in part due to the disruption of mother–infant interaction (37,46,47). First of all, there is evidence that maternal behavior towards the pups is sex dependent, i.e., male and female pups are exposed to different patterns of maternal care (1,24,25). It can be hypothesized that male and female pups miss out on different components of maternal care during MS, and that after reunion rat dams do not care in the same way for both sexes, which may lead to different long-term effects. Additionally, males and females may differ in their HPA axis development, because steroid hormones influence neural pathway organization in the brain during development (4) and, therefore, a separation on PND 4 may lead to different effects in males and females due to different levels of HPA axis plasticity.

In this study we found consistent sex differences in our control subjects (on the majority of paradigms used) that were in the same direction as those reported in numerous studies in the literature. This agreement with the generally described and accepted data on sex differences serves as a validation of our study and of the evidence that we report for the rather marginal effects of MS. Against this validation, it is apparent that MS conducted at each of the three developmental stages used here does not exert a consistent effect on unconditioned fear/anxiety and aversive conditioning in adulthood. At the same time, it is potentially important that some specific effects of MS were obtained: most notable in this respect is the marked acquisition deficit in active avoidance in male subjects at PND4 and the superior rule reversal learning in the water maze task in all MS groups.

In summary, the present study has provided a comprehensive analysis of the hypothesis that MS during a period of 24 h will exert a significant impact on spontaneous fear/anxiety and on conditioning in an aversive situation, and that effects of the manipulation will be enhanced in males relative to females, and enhanced or even opposite when performed early in the SHRP (age 4 days) relative to later in (9 days) or subsequent to (18 days) the SHRP. The evidence that we obtained clearly indicates that MS at any of the three stages does not affect unconditioned fear/anxiety in males or females, but that MS early in the SHRP does tend to disrupt aversive conditioning, particularly in males, and that MS later in the SHRP—if it has any effect at all—tends to enhance aversive conditioning.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Swiss Federal Institute of Technology Zürich. We thank the Animal Services for the husbandry and care of the animals, Peter Schmid for technical support and Bonnie Strehler for assistance with preparation of the manuscript.

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