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Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long–Evans rats

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Abstract

Early neonatal environmental factors appear to have powerful and long-lasting influences on an organism's physiology and behavior. Long–Evans male rats separated from their dam for 3 h daily over the first 2 weeks of life (maternally separated, MS rats) when tested as adults exhibit exaggerated behavioral and neuroendocrine responses to stress compared to 15-min separated (handled, H) animals. The purpose of this study was to compare male and female adult rats that were MS, H or were undisturbed (nonhandled, NH) as neonates in anxiety-like behaviors, in the elevated plus-maze, and in response to startle-inducing auditory stimuli. We confirmed that MS males oversecrete corticosterone (CORT; 2.5–5 times) in response to mild handling stress. MS males and females were less likely to explore open arms of the plus-maze. MS males exhibited 35% higher startle amplitudes compared to controls. Furthermore, MS males were more likely to emit ultrasonic vocalizations in response to startle than were H controls. However, MS and control females did not differ in auditory startle response or in startle-induced ultrasonic vocalizations. Therefore, experiencing maternal separation results in a long-lasting increase in anxiety-like behaviors that occurs in a sex-dependent manner.

Keywords: Maternal separation; Fear and anxiety; Elevated plus-maze test; Auditory startle response; Ultrasonic vocalization; Sex differences

1. Introduction

The last few decades have seen a tremendous increase in interest in the topic of early environmental influences on an organism's physiology and behavior. A large body of evidence indicates that exposure to early adverse life events in the form of childhood neglect and abuse can increase vulnerability to psychopathology in adult life (Heim et al., 1997, 2000; Ladd et al., 2000; Caldji et al., 2001).

Periodic neonatal maternal separation in the rat has been used by several investigators as a rodent model of the effects of early adverse life events on adult physiology and behavior. In this procedure, neonatal rats are removed from the mother for several hours daily during the first 2 weeks of life (Plotsky and Meaney, 1993; Wigger and Neumann, 1999; Kalinichev et al., 2000; Huot et al., 2001; Boccia and Pedersen, 2001). This procedure is based on the observation by Calhoun (1963) that, in a seminaturalistic environment, subordinate dams are often forced to build their nests at some distance from food and water resources. This results in extension of mother–litter separations from typical 15–30 min to as long as 2–3 h (Francis et al., 1999; Liu et al., 2000). When tested as adults, maternally separated (MS) adult offspring exhibit a cluster of behavioral and neuroendocrine signs similar to those observed in patients with depression and anxiety disorders (Amsterdam et al., 1987; Heit et al., 1997; Ladd et al., 2000). A primary feature of the MS phenotype has been an enduring dysregulation of hypothalamic–pituitary–adrenal (HPA) axis reactivity to stress (Ladd et al., 2000; Caldji et al., 2001). Compared to males that were either separated daily for 15 min (handled, H) or completely undisturbed (nonhandled, NH) as neonates, MS males have elevated basal CRF levels in the median eminence and elevated levels of the CRF mRNA levels in the hypothalamus (Plotsky and Meaney, 1993). In response to stress, such as restraint or air-

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puff startle, MS males display a potentiation in ACTH and corticosterone (CORT) secretion compared to either H (restraint) or H and animals that received typical animal husbandry (animal-facility reared, AFR; air-puff; Plotsky and Meaney, 1993; Huot et al., 2001). Furthermore, in MS males, there is significant reduction of $GABA_A$ receptor levels in the medial prefrontal cortex, as well as in locus coeruleus and nucleus tractus solitarius (Caldji et al., 2000). This decrease in GABA-binding sites might result in potentiated noradrenergic stimulation of CRF synthesis and release (Plotsky et al., 1989) and contribute to increased CORT and ACTH responses to stress observed in MS animals (Liu et al., 2000; Huot et al., 2001). In addition, compared to H and NH males, MS males have increased stress-induced norepinephrine responses in the paraventricular nucleus of the hypothalamus, a major source of the CRF that regulates the HPA activity (Liu et al., 2000).

In accord with the neuroendocrine changes, there is some evidence of behavioral alterations in MS animals that resemble those observed in patients with affective disorders. MS rats appear to exhibit increased anxiety-like behavior, anhedonia, increased preference for ethanol and impairment in male sexual behavior (Wigger and Neumann, 1999; Caldji et al., 2000; Ladd et at., 2000; Huot et al., 2001; Rhees et al., 2001).

While the majority of investigators examined the effects of maternal separation only in male offspring, those that studied both males and females found sex-dependent effects. For example, while MS and NH males are less likely than H males to explore open arms of the plus-maze and consume a novel food, MS females resemble H females, exhibiting decreased anxiety and neophobia compared to NH females (McIntosh et al., 1999). According to Wigger and Neumann (1999), the impact of maternal separation in decreasing open-arm activity in the elevated plus-maze is more robust in males than in females. Also, exposure to the plus-maze test potentiates the ACTH release in MS males but not in MS females (Wigger and Neumann, 1999). We found that MS males are less sensitive to morphine than are H and NH males in the hot-plate test of antinociception, while this difference is absent among the female groups (Kalinichev et al., 2001).

The increased interest in the paradigm of maternal separation by several laboratories also has resulted in discrepancies among findings, especially concerning behavioral signs of anxiety in MS animals (Lehmann and Feldon, 2000). Some of the differences in outcomes among studies may be, in part, due to the diversity of separation procedures used (e.g., different strains, duration and days of separation, whether the offspring are isolated individually or kept as a litter, ages at testing and behavioral tests applied). For example, Sprague–Dawley males that were separated from their mother for 6 h daily on postnatal days 2–10 when tested as adults did not differ from NH males in the open-field activity (Rhees et al., 2001). In contrast, Sprague–Dawley males separated from the mother for 4.5 h daily during the first 3 weeks of life were less active in the open-field test compared to NH males (Ogawa et al., 1994).

Another source of discrepancy appears to be the control group that is used for a comparison with MS animals. Overall, there is an agreement that MS males of several rat strains exhibit signs of increased anxiety compared to H males in the open-field, plus-maze, auditory startle and novelty-induced suppression of feeding tests (McIntosh et al., 1999; Wigger and Neumann, 1999; Caldji et al., 2000; Huot et al., 2001). However, there is lack of agreement on whether MS animals exhibit signs of increased anxiety compared to animals that were completely undisturbed as neonates (NH group). According to some authors, MS males exhibit increased anxiety compared to NH males, as indicated by decreased open-field activity and increased likelihood of approaching food in a novel arena when food deprived (Ogawa et al., 1994; Caldji et al., 2000). However, in other measures of novelty-induced suppression of feeding and in the plus-maze test, MS and NH males are similar (McIntosh et al., 1999; Caldji et al., 2000). Furthermore, plasma levels of CORT and ACTH following stress are also similar in MS and NH males (Plotsky and Meaney, 1993; Liu et al., 2000). According to others, plasma CORT levels during (Ogawa et al., 1994) or following (Pryce et al., 2001) restraint stress are even higher in NH than in MS males.

The primary goal of our study was to shed some light on the MS vs. H vs. NH comparison in anxiety-like responses in both males and females. In particular, elevated plus-maze activity and startle-induced ultrasonic distress vocalizations were studied in Long–Evans rats that experienced 3-h daily separations from the dam during the first 2 weeks of life. Initially, we wanted to confirm that, under our experimental conditions, stress-induced secretion of CORT and ACTH is significantly higher in MS males compared to H males (Plotsky and Meaney, 1993; Liu et al., 2000; Huot et al., 2001). We hypothesized that repeated separation from the mother would result in significant alteration in the offspring's reactivity to aversive environmental stimuli in a sex-dependent manner.

2. General methods

2.1. Subjects

The subjects were male and female Long–Evans hooded rats (Blue-Spruce, Harlan Sprague–Dawley, Indianapolis, IN) 120 days of age at the time of testing. All the subjects were the offspring of dams that were shipped to our facility on their 12th day of pregnancy. The pups were born and raised in our colony. A total of 108 males and 48 females were used in this study. Food and water were always available ad libitum. The colony room was maintained on a 12:12 light–dark cycle with lights on at 0700 h. These studies were performed in full accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health; the research protocol was approved by the Institutional Animal Care and Use Committee of Emory University.

2.2. Maternal separation procedure

We used a procedure for mother–infant separation that has been shown by several laboratories to result in robust behavioral and neuroendocrine differences between MS and control offspring (Plotsky and Meaney, 1993; Caldji et al., 2000; Liu et al., 2000; Huot et al., 2001; Boccia and Pedersen, 2001). Pups that composed 30 original litters were taken from their dams, randomized and redistributed among the dams on postpartum day 2 (date of birth=day 1) as all-male (eight pups) litters (seven litters per separation group; Plotsky and Meaney, 1993). In addition, a few of the dams (three litters per separation group) received all female (10 pups) litters, approximating the total weight of the male litter. Random redistribution of pups among dams was done in order to eliminate possible effects of genetic and prenatal factors. For maternal separation, an entire litter was removed from the foster dam, which remained in the home cage, and placed into a bedding-lined plastic container in an incubator (Veterinary water-lined warmer, ThermoCare, Incline Village, NE) maintained at 31 °C. No more than two animals from the same rearing litter were used in each experimental group. MS litters were kept in the incubator for 3 h and then returned to their foster dam. This occurred daily between 0900 and 1200 h on postnatal days 2–14. H animals experienced a similar procedure, however, the pups remained away from their foster dam for only 15 min. NH animals were returned to the foster dam immediately after group assignment (postnatal day 2) and left undisturbed until weaning on day 22. Our rationale for including both H and NH groups for the comparison with MS animals was the following. While robust differences between MS and H males have been demonstrated, there is a lack of conclusive evidence of differences between MS and NH, in males and especially in females in anxiety-like behavior. In addition, we wanted to replicate the paradigm used in previous work of one of the authors (Plotsky and Meaney, 1993; Liu et al., 2000; Caldji et al., 2000). After weaning, animals were group-housed in same-sex, same-treatment-group cages. Initially, there were four littermates per single cage. After animals reached 120–150 g, they were further separated into two animals per single cage.

2.3. Blood sampling procedure

Based on preliminary evidence that the HPA axis of MS males can be sensitive to perturbations associated with even a light stress (activity on the plus-maze, air-puff startle; Wigger and Neumann, 1999; Huot et al., 2001), brief exposure to light handling was chosen as a stressor.

Twenty-four male rats (n=8/group) were used for this experiment. Each animal was gently handled for 2–3 min while being transferred into a different room and decapitated. Trunk blood was collected, centrifuged and plasma was stored at −40 °C until analysis. Plasma ACTH and CORT were determined, using commercially available radioimmunoassay assay kits (Plotsky et al., 1992; Thrivikraman et al., 1997). ACTH was assayed in 100-μl plasma samples by the Allegro HS-ACTH kit (Nichols Institute, San Juan Capistrano, CA), using 50 μl of iodinated tracer and one avidin-coated bead per tube. Sensitivity was 1 pg/tube, with an EC50 of 15 pg (working range 1–1500 pg/ml). The CORT assay was performed using the ImmuChem Double Antibody kit (ICN Biomedicals, Costa Mesa, CA), which was modified by reducing the volume of trace and antibody added to each assay tube by half of the suggested (kit insert) volume. The working range for CORT was 5–1000 ng/ml with <7% intraassay coefficients of variation for either assay.

2.4. Elevated plus-maze test

The elevated plus-maze was made of opaque plastic and was 60 cm off the floor. It had four arms radiating outward from a central open square (10×10 cm). Two were open-sided runway-style arms (50×10 cm) and two were enclosed. The floor of the enclosed arms was the same size as the open, but these arms had sidewalls 40 cm high.

Thirty-six male (n=12/group) and 24 female (n=8/group) subjects were used in this experiment. Each rat was placed in the plus-maze, facing a closed arm, and was allowed to explore freely the plus-maze for 10 min. During the test, the following parameters were recorded: (a) entries into open arms, (b) time spent on open arms and (c) overall activity (entries into closed arms). Each animal was tested twice, in dim and in normal light conditions. The sequence of the testing was randomized across animals. Under dim light conditions, a single red bulb was used (18 lx). Under normal light conditions, the testing room was illuminated by standard fluorescent lights at 350 lx (adjusted for the fluorescent bulb), as measured by a Dual Range Digital Light Meter (VWR Scientific, Model 62344-944, Control, Friendswood, TX) placed on one of the open arms of the plus-maze.

2.5. Acoustic startle response test

The acoustic startle response (MED Associates, St. Albans, VT) was measured in sound-attenuating cubicles by recording the force applied by a startled rat onto a single point load cell (the startle transducing platform). Rats were placed into Plexiglas cylinders (internal measures: 14 cm long × 7.5 cm diameter) fixed on top of the startle platform (25×11 cm). A 55-dB background white noise was constantly maintained inside the cubicles. Startle stimuli were delivered after a 3-min acclimation period. Speakers were placed approximately at the level of the rat's ears. The startle stimulus was white noise. The startle response was recorded

Table 1
Body weights (g) at the last day of separation (postnatal day 14) and into adulthood (postnatal days 30, 60 and 90) of Long–Evans male and female rats that were MS or were H or NH

		14 days	30 days	60 days	90 days
Males	NH	31.7 ± 0.4	102.3 ± 1.7	330.7 ± 4.9	425.4 ± 8.5
	H	30.7 ± 0.4	101.8 ± 1.2	329.8 ± 6.7	427.6 ± 6.4
	MS	28.7 ± 0.5*	101.1 ± 1.7	346.2 ± 5.0	434.7 ± 8.3
Females	NH	27.2 ± 0.5	89.5 ± 1.4	219.3 ± 3.0	265.6 ± 5.1
	H	26.1 ± 0.6	90.2 ± 1.5	226.1 ± 4.7	270.9 ± 3.2
	MS	28.9 ± 0.6*	88.4 ± 1.6	221.0 ± 4.6	266.6 ± 5.6

* Significantly ($P<.05$) different from H and NH animals.

during the 500 ms following the onset of the startle stimulus. Acoustic startle peak was defined as the first peak of the downward force that was produced by the rat on the platform, with the minimum latency −20 ms, minimum peak value 50 (range 50–2047) and minimum peak time 30 ms. The upward response of the platform was recorded but was not analyzed. All events during the startle session were recorded and controlled by Med Associates software (Version 3, Startle Reflex). Before the start of the experiment, all auditory stimuli were calibrated (precision of 0.5 dB) in each box, using the Digital Sound Level Meter (Model 840029, Sper Scientific, Scottsdale, AZ). Each startle platform was calibrated by using a constant force produced by a weight displaced around a 45° angle by a rotating-step motor. Four startle platforms varied by <2% in the startle calibration procedure. We calibrated the amplifier gain that fed the analog-to-digital converter independently for males and for females due to the difference in body weight.

In a pilot experiment, separate groups of rats (n=10–20/group/sex) were exposed to 18 discrete (30 ms) auditory pulses of varying intensities (90, 105 and 120 dB), presented in a random order, with the intertrial interval of 30–45 s. Because the 90-dB stimulus produced inconsistent startle responses and the 120-dB stimulus produced near-maximal startle amplitudes (data not presented), 105-dB stimuli were chosen for the subsequent tests. The startle test session (approximately 17-min long) consisted of exposure to 18 (3 blocks of 6 trials) discrete (30 ms) auditory pulses (0 ms rise/fall time) of the same intensity (105 dB) with the intertrial interval of 30–45 s.

2.6. Ultrasonic vocalizations

During the startle test sessions, ultrasonic vocalizations (20–28 kHz) were recorded (Mini-3 Bat Detector, Ultra Sound Advice, London, UK) and transformed into an audible signal (0.2–10 kHz). With the aid of an audio filter (Noldus Information Technology, Sterling, VA), the signal was then sent to a computer, digitized and analyzed by the computer with the aid of the software UltraVox (Noldus Information Technology, Sterling, VA).

2.7. Statistical analysis

The homogeneity of variance of the interval scale data was evaluated using Bartlett's test. The interval scale data that met the requirements for parametric statistical procedures were analyzed by the analysis of variance (ANOVA) in order to determine the main effect. Fisher's protected least significant difference or Scheffe's post-hoc test was used for multiple comparisons. The interval scale data that did not meet the requirements for the homogeneity of variance (duration on open arms of the plus-maze) were analyzed by the Kruskal–Wallis test. The Mann–Whitney *U* post-hoc test was used for multiple comparisons. All proportion data (percentage of animals entering open arms of the plus-maze, percentage of animals emitting ultrasonic vocalizations) were analyzed with the chi-square test. In all statistical tests, the α level chosen was $P<.05$.

3. Results

3.1. Body weights

Group means (with standard errors) of body weights of MS and control animals on postnatal days 14, 30, 60 and 90 are presented in Table 1. At the age of 14 days, the last day when the mother–litter separation was performed, MS males were significantly (up to 10%) lighter in body weight

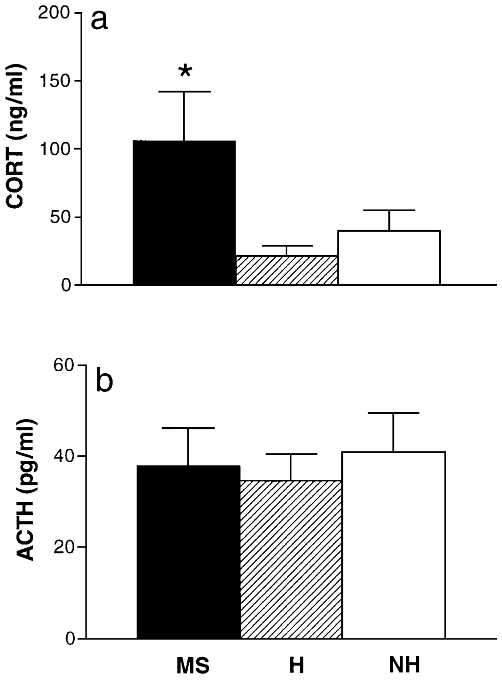

Fig. 1. (a–b) Plasma CORT (ng/ml) and ACTH (pg/ml) immediately following brief mild handling stress in MS, H and NH males. Mean (+S.E.M.; n=8/group). *$P<.05$ compared to H and NH males.

compared to both H and NH animals [$F(2,32)=12.7$, $P<.0001$; Table 1]. At the same age, MS females were significantly (up to 10%) heavier compared to both H and NH females [$F(2,29)=6.4$, $P<.01$; Table 1]. Beginning at the age of 30 days, experimental groups within both sexes were no longer different in body weight (Table 1).

3.2. Validity of the HPA hyper-responsiveness in MS animals

Exposure to mild stress of handling resulted in plasma CORT levels that were significantly higher in MS males compared to either H or NH males [$F(2,20)=3.38$, $P=.05$; Fig. 1a]. In contrast, plasma levels of ACTH did not differ in MS, H and NH males (Fig. 1b).

3.3. Elevated plus-maze test

3.3.1. Males

Under dim lighting conditions, 83–100% of males entered the open arms of the plus-maze (Fig. 2a). In duration of time spent on open arms, the overall group differences approached but did not reach statistical significance [$H(df=2)=4.65$, $P=.09$]. Planned comparisons revealed that H males spent less time on open arms than did NH males

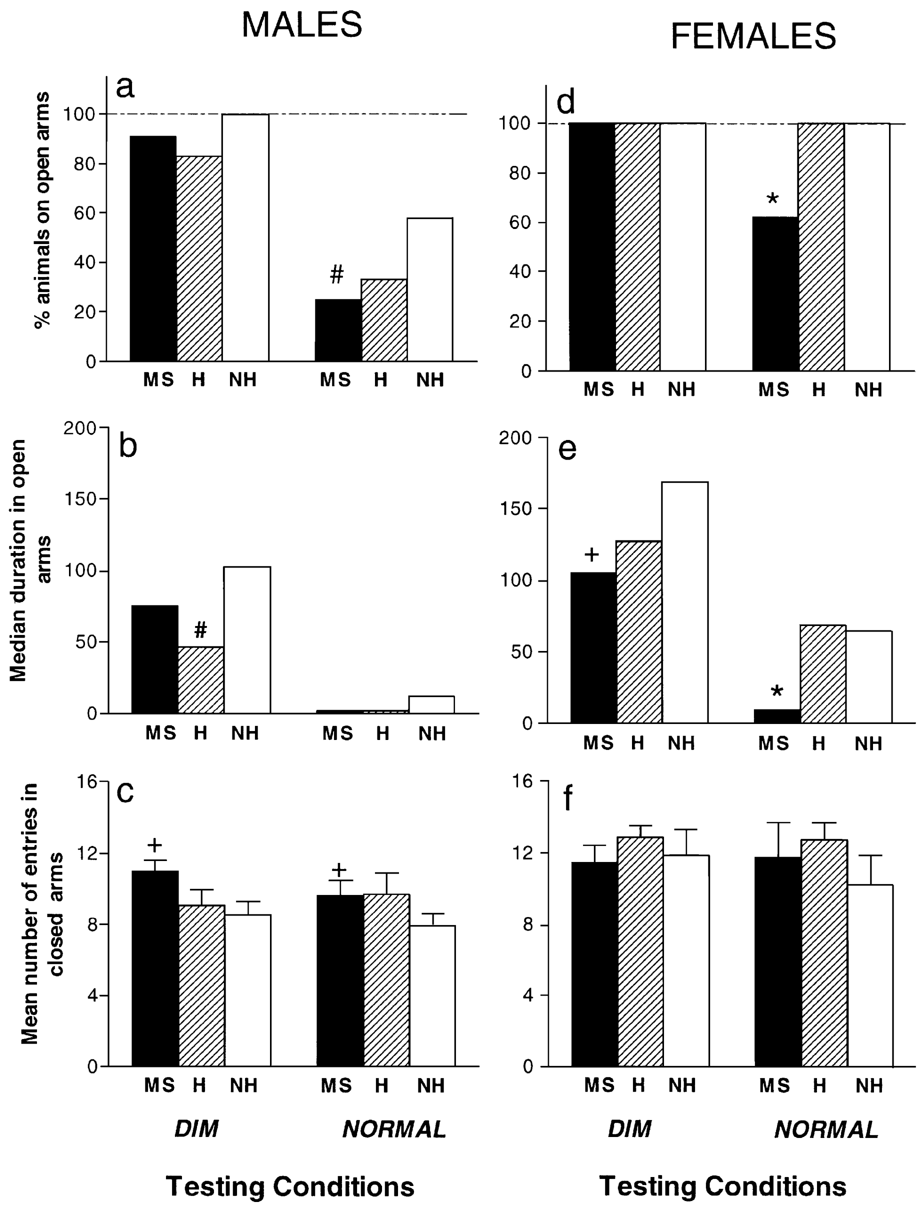

Fig. 2. (a–f) Performance on the elevated plus-maze of the MS, H and NH male (a–c) and female (d–e) rats in dim and in normal light condition. Percentage of animals exploring open arms (a,d). Median ($n=8-12$/group) duration (s) exploring open arms (b,e). Mean (+S.E.M.; $n=8-12$/group) number of entries into closed arms (c,f). $^*P<.05$ compared to H and NH groups; $^+P<.05$ compared to NH animals; $^\#.05<P<.1$ compared to NH males.

(.05<P<.1; Fig. 2b). Testing under normal light decreased the percentage of males entering open arms by 66% in MS males and by 42–50% in H and NH control animals (Fig. 2a). In normal light, a nonsignificant trend was detected in that fewer MS males (only 25%) entered open arms compared to NH control animals [almost 60%; χ^2 (1,n=24)=2.74, P=.09]. The duration of time spent on open arms was very low in all males (median 0–12 s) and did not differ across groups (Fig. 2b). The overall group differences in the number of entries to closed arms, across light conditions were statistically significant [F(2,33)=3.29, P<.05; Fig. 2c]. Planned comparisons revealed that MS males entered closed arms significantly more often than did NH males (P<.05; Fig. 2c).

3.3.2. Females

In dim light, all females entered the open arms of the plus-maze (Fig. 2d). There were significant overall group differences in the duration of time spent on open arms [H(df=2)=6.68, P<.05; Fig. 2e]. Pairwise comparisons revealed that MS females spent significantly less time on open arms than did NH controls (P<.05; Fig. 2e). When females were tested under normal light conditions, significantly fewer (only 60%) of the MS group entered open arms compared to 100% of control (H and NH) females [χ^2 (2,n=24)=3.69, P<.05; Fig. 2d]. Furthermore, MS females spent significantly less time in open arms compared to either H or NH controls [H(2)=6.46, P<.05; Fig. 2e]. Under both testing conditions, MS, H and NH females were similar in the number of entries to closed arms (Fig. 2f).

3.4. Acoustic startle response

3.4.1. Males

The peak values of the auditory startle were consistently and significantly (35%) higher in MS males compared to either of the control groups (H and NH) in response to all three (six-trial) blocks of 105-dB startle stimuli [F(2,90)=3.43, P<.05; Fig. 3a]. Furthermore, there were significant differences across groups in latencies to startle in response to the first two bocks of 105-dB startle stimuli [F(2,45)=4.52, P<.05; Fig. 3b]. This difference was due to the fact that MS males had significantly (P<.05) shorter startle latencies compared to males in H group, and showed a nonsignificant trend toward a similar difference from NH control animals (P=.08).

3.4.2. Females

The peak values of the auditory startle were similar across MS, H and NH females throughout the testing (Fig. 3c), as were startle latencies (Fig. 3d).

3.5. Ultrasonic distress vocalizations test

Exposure to the series of 105-dB acoustic stimuli induced ultrasonic distress vocalizations in number of animals. As a nonsignificant trend, MS males were more likely (37%) to emit ultrasonic vocalizations in response to auditory startle stimuli than were H males [12%; χ^2 (1,n=36)=2.67, P=.09; Fig. 4a]. The percentage of NH males emitting ultrasonic vocalizations (25%) did not differ from that of either the MS or H groups (Fig. 4a). In contrast

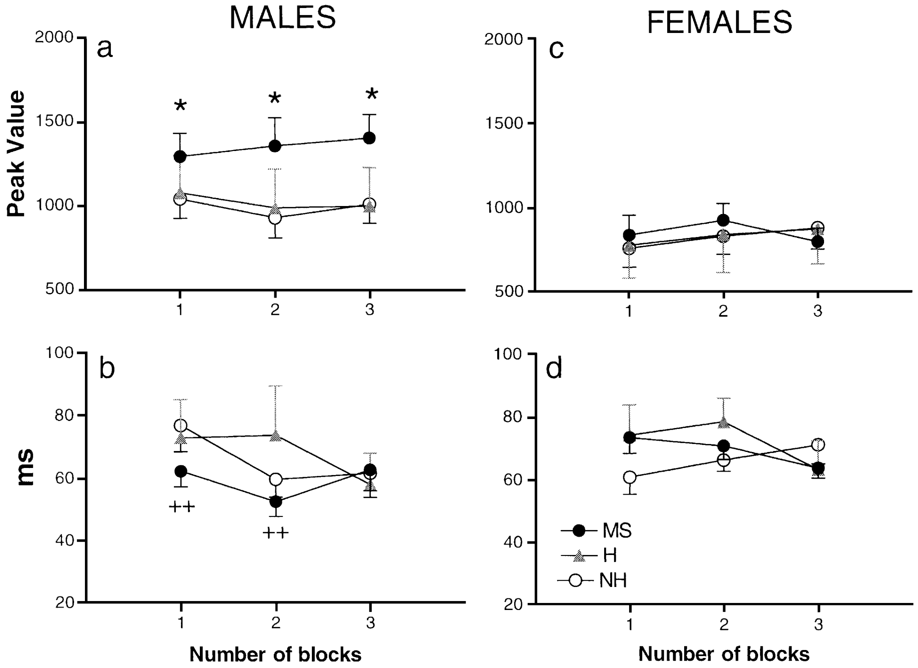

Fig. 3. (a–b) Performance in response to loud auditory stimuli of the MS, H and NH males (a,b) and female (c,d) rats. Mean (+S.E.M.; n=16/group in males; n=8/group in females). *P<.05 compared to H and NH males; ^{++}P<.05 compared to H males.

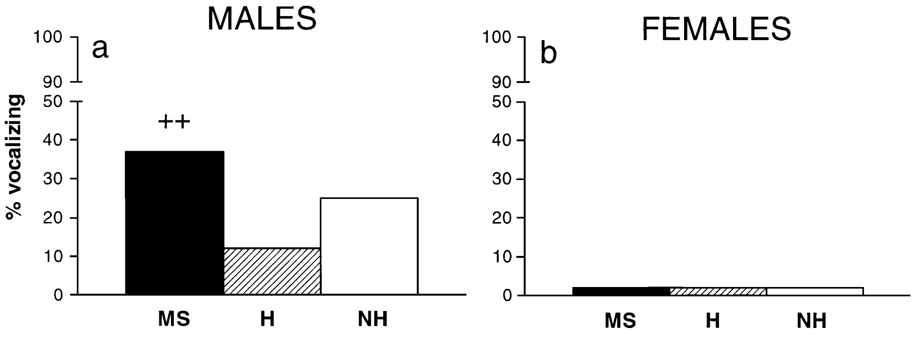

Fig. 4. (a–b) Ultrasonic distress vocalizations induced by loud auditory stimuli in MS, H and NH control males (a) and female (b) rats. $^{++}.05<P<.1$ compared to H males.

to males, exposure to the series of 105-dB auditory stimuli failed to induce ultrasonic distress vocalizations in any females (Fig. 4b).

4. Discussion

In Long–Evans rats, repeated 3-h separation from the mother during the first 2 weeks of neonatal life resulted in enhanced secretion of CORT in response to mild stress and in altered anxiety-like behaviors in response to aversive environmental stimuli. Whether a particular environmental challenge (i.e., test for anxiety-like behavior) can reveal behavioral differences between MS, H and NH animals appears to be influenced significantly by the sex of an animal.

Under normal conditions, during the first 2 weeks following birth of the offspring, a lactating female rat leaves the maternal nest regularly. Voluntary separations from the litter can last for a period of 10 min to 1 h, depending on the age of the offspring (Grota and Ader, 1969). Under natural and seminatural conditions, dominant female rats build their nests in close proximity to food/water resources and keep regular separations from the offspring relatively brief. However, subordinate females are forced to build their nests in the periphery, resulting in significantly longer foraging periods and therefore longer periods of maternal separation lasting as long as 2–3 h (Calhoun, 1963). In an experimental setup, daily, brief (15 min) separations from the litter ("handling") facilitate pup-licking and arched-back nursing in Long–Evans dams (Lee and Williams, 1974; Liu et al., 2000), thereby providing support to the maternal-mediation hypothesis (Denenberg, 1964). Whether or not prolonged (> 3 h) separations result in opposite effect—a deficit in maternal care—requires further investigation. According to some authors, Long–Evans dams that raise MS litters are slower to initiate pup retrieval and other maternal behaviors relative to H and AFR dams (Huot et al., 1997). A deficit in the frequency of pup grooming also has been observed (Boccia and Pedersen, 2001). In contrast, according to Pryce et al. (2001), 4-h separated Wistar dams exhibit a transient increase in licking and arched-back nursing in the first hour after reunion. Regardless of their effect on maternal responsiveness, 3-h separations result in enduring changes in fear/anxiety-related behaviors and sensitivity to morphine in the dam (Kalinichev et al., 2000). In humans, prolonged lack of contact between a mother and her infant soon after birth results in significant deficits in maternal attachment (Leifer et al., 1972).

As an indirect measure of maternal deficit, on the last day of the separation procedure (postnatal day 14), MS males weighed significantly (7–10%) less than did either H or NH males (Table 1). In full accord, neonatal maternal separation of Sprague–Dawley male rats for 3–4.5 h daily resulted in lower body weights at weaning on day 22 (Ogawa et al., 1994; McIntosh et al., 1999). While others (McIntosh et al., 1999) described a similar deficit in MS females, in our study, MS females were significantly (up to 10%) heavier than either H or NH females. Deviations in body weight exhibited by MS animals appeared to be transient; by the age of 30 days, differences in body weight across the groups had disappeared in both males and females. Other investigators, however, have found that the weight deficit of MS animals can be still present at 30 days of age (Rhees et al., 2001) and can even persist well into the adulthood (Ogawa et al., 1994; McIntosh et al., 1999; Huot et al., 2001).

Exposure to even the mild stress of brief handling resulted in dramatically higher plasma CORT levels in MS males compared to either H (5 times) or NH males (2.6 times), which were equally low. Unfortunately, the design of the experiment excluded the possibility of taking blood samples repeatedly, before (baseline) and after the handling stress. These findings complement those by others indicating that MS males exhibit higher stress-induced CORT levels compared to H and AFR males (Plotsky and Meaney, 1993; Huot et al., 2001). In contrast to our findings, plasma CORT levels in NH males were found to be as high as in MS males (Plotsky and Meaney, 1993) or even higher (Ogawa et al., 1994; Pryce et al., 2001). It has been hypothesized that the neonatal NH experience yields a phenotype that resembles the MS group, with increases in anxiety-like behavior and in HPA reactivity to stress (Levine, 1957; McIntosh et al., 1999; Caldji et al., 2000; Pryce et al., 2001). Our present data (both HPA and behavioral) and some evidence from other laboratories

(Ogawa et al., 1994; Rhees et al., 2001) do not support this hypothesis. What are the environmental factors (e.g., degree of nondisturbance) during the neonatal period that are necessary to yield the MS-like phenotype in NH animals? This interesting issue needs further investigation. It is still surprising that, even though plasma CORT levels in H males were approximately half of those detected in NH males, there was no statistical difference between these groups. Perhaps, factors associated with intensity/duration or aversiveness of the stress, while revealing altered sensitivity of the MS group, were suboptimal for detecting H vs. NH differences. The same factors may, in part, be responsible for the lack of differences in stress-induced ACTH levels among the groups. It is perplexing that there was a significant increase in plasma CORT levels, while levels of ACTH did not exceed a typical basal range. Additional studies evaluating the effect of low-intensity or psychological stressors could be fruitful for understanding phenotypic differences between MS, H and NH animals.

The elevated plus-maze test, assessing internal conflict between voluntary approach and withdrawal tendencies, has been used widely as a rodent model of human anxiety (Pellow et al., 1985; Cruz et al., 1994; Rodgers and Dalvi, 1997). We tested animals under two conditions that presumably differed in the degree of their aversiveness, in dim light and in normal (fluorescent) light. In dim light, almost all of the animals (85–100%), regardless of their sex, entered open arms of the plus-maze; testing in normal light increased avoidance of open arms primarily by MS animals. When tested in normal light, only 25% of MS males vs. 60% of NH males entered open arms of the plus-maze. Increased anxiety in the MS phenotype compared to the NH group was even more pronounced in females. MS females was the only group in which there was a decrease in the percentage (40%) of animals entering open arms of the plus-maze when tested in normal light conditions. As an additional indication of an increase in anxiety-like state, MS females spent significantly less time exploring open arms of the plus-maze compared to NH females under both dim and normal light. In accord, Wistar rats that experienced maternal separation (males and females) exhibited decreases in open-arm exploration compared to AFR controls that received normal husbandry as neonates (Wigger and Neumann, 1999). Also, in Long–Evans rats, MS males exhibited decreases in open-arm exploration compared to H and AFR males (Huot et al., 2001). In contrast to our expectations, compared to MS and NH groups, H animals did not exhibit decreases in anxiety-like behavior in the elevated plus-maze test. H females overall resembled NH females in open-arm activity, whereas H males spent even less time on these arms than NH males did. While several investigators (Nunez et al., 1995; Vallee et al., 1997; Ploj et al., 1999) described increases in open-arm activity in H animals, others were unable to detect similar effects (Wakshlak and Weinstock, 1990; Durand et al., 1998). According to Durand et al. (1998), compared to NH experience, neonatal handling failed to influence open-arm activity in male and female Lewis rats (Durand et al., 1998). Also, in Long–Evans rats, H males exhibited signs of decreased anxiety compared to MS males, but were similar to AFR males (Huot et al., 2001). It is possible that factors that influence the outcome of maternal separation are also relevant for the handling effect. The general motor activity assessed by the number of entries into closed arms of the plus-maze was not influenced by the light conditions, either in males or in females. General activity was higher in MS males compared to NH males, complementing similar findings by us (Kalinichev et al., in review) and by others (Pryce et al., 2001).

While in the elevated plus-maze, an animal is tested for voluntary exploration of an aversive environment; in the acoustic startle response test, an animal is challenged by an acute, uncontrollable stress (loud noise). While females did not differ in auditory startle, MS males were more responsive (higher amplitudes) and were quicker to respond than H and NH males. In accord, 1-h separations on neonatal days 2–11 also resulted in increased startle amplitude in separated Wistar rats (Finamore and Port, 2000). However, more prolonged (6 h) separations later in neonatal life (postnatal days 12, 14, 16 and 18) failed to have an effect on the auditory startle response (Lehmann et al., 2000). Perhaps, maternal separation performed during the late neonatal period has a lesser impact on subsequent behavior. Starting around day 12, neonatal rats are more independent from the dam and begin to spend more time away from her (Rosenblatt and Lehrman, 1963). In the Long–Evans rats used in this study, there was no detectable habituation in acoustic startle response, regardless of the sex or the neonatal experience. In contrast, startle habituation in response to similar repetitive auditory stimuli has been described in Sprague–Dawley (Pilz and Schnitzler, 1996) and Wistar (Lehmann et al., 2000) rats. Because there can be significant strain differences in startle habituation and amplitude (Pilz et al., 1999), Long–Evans rats may be characterized by delayed habituation to particular auditory stimuli compared to other strains.

Similar to some other types of aversive stimuli, such as attacks from conspecifics (Sales, 1972) or electric footshocks (Van der Poel et al., 1989), startle-inducing acoustic stimuli induce rats to emit ultrasonic (20–28 kHz) distress vocalizations (Kaltwasser, 1990; Van der Poel and Miczek, 1991; Miczek et al., 1995; Kalinichev et al., 2000). According to several studies, clinically active anxiolytic drugs decrease ultrasonic distress vocalizations in the rat (Tonoue et al., 1987; Kaltwasser, 1991; Vivian et al., 1997). In overall accord with the plus-maze and auditory startle data, MS males exhibited signs of increased anxiety (37% of animals vocalizing) compared to H or NH males (12–25% vocalizing). While the elevated plus-maze test revealed robust differences among MS, H and NH females in anxiety-related behaviors, acoustic startle and ultrasonic vocalization tests failed to detect such differences. Even though both elevated plus-maze and ultrasonic vocalizations tests are designed to model human anxiety in rodents, in some cases, an opposite effects in the two tests have been observed (Kalinichev et al., 2000; White et al., submitted). However, there is growing evidence that measures from different animal tests reflect different types or aspects of anxiety (File, 1991, 1996; Belzung and Le Pape, 1994) and, presumably, different neuronal circuitry.

In our study, we followed a maternal separation procedure (Plotsky and Meaney, 1993) in which rat pups are cross-fostered into all male or all female litters. There is some evidence that, in a mixed litters, Long–Evans dams lick their male pups more often and for longer period than female pups (Moore and Morelli, 1979; Moore et al., 1997). There is a possibility that same-sex litters can alter mother–infant interactions and therefore can influence the outcome of maternal separation or handling in adulthood. However, some of the key effects of maternal separation and handling have been observed in other rat strains as well as having been replicated in Long–Evans rats, regardless of the litter design (Plotsky and Meaney, 1993; Huot et al., 2001).

To summarize, periodic 3-h separations from the dam during the neonatal period had robust and enduring effects on physiology and behavior of Long–Evans rats. Compared to NH males, MS males exhibited exaggerated CORT responses to mild stress and signs of increased anxiety in response to a variety of environmental stimuli. Expression of increased anxiety in MS females appeared to be test-dependent. Animals that experienced periodic 15-min separations as neonates (H animals) failed to exhibit signs of decreased anxiety or HPA reactivity to stress typically associated with this phenotype. The environmental factors that mediate the expression of MS, H and NH phenotypes in adulthood require further exploration.

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