TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2016) 46: 1546-1560

doi:10.3906/sag-1502-39

Adulthood behavioral and neurodevelopmental effects of being raised by an ambivalent mother in rats: what does not kill you makes you stronger

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Received: 11.02.2015 • **Accepted/Published Online:** 13.12.2015 • **Final Version:** 17.11.2016

Background/aim: This study aimed to investigate the effects of early adverse life events and being raised by an ambivalent mother on rats.

Materials and methods: The rats were separated into four groups: 1) the control group (n = 12), which was raised under standard care; 2) the early handling (EH) group, which was raised using an EH model (n = 16); 3) the early deprivation (ED) group, which was raised using an ED model (n = 13), and 4) the ambivalent mother (AM) group, which spent 3 h/day with a "fake mother" (n = 17). When they became adults, their anxiety levels, depressive-like behaviors, and memory functions were measured using the elevated plus maze test, the forced swim test, and the novel object recognition test, respectively. Their neurodevelopment was evaluated by measuring the brain-derived neurotrophic factor (BDNF) levels in the prefrontal cortex, the dentate gyrus, and the cerebellum via ELISA.

Results: The rats in the ED and AM groups exhibited less anxiety and depressive-like behavior than those in the control and EH groups, particularly in females. There was no significant difference between the groups in memory function or brain BDNF levels.

Conclusion: Severe and ambivalent early adverse life events may decrease anxiety and depressive-like behavior in adult rats.

Key words: Ambivalent mother, early adverse life events, behavioral analysis, anxiety, depression, memory, neurodevelopment, rats

1. Introduction

Early adverse life events result in a high risk for the development of many psychiatric illnesses, such as depression, personality disorders, substance dependence, and schizophrenia, in adulthood (1,2). Human studies investigating the long-term effects of early adverse life events include two major limitations: one cannot exclude the genetic effects, and there is a risk of false memories. Thus, efforts have been made to develop animal models of early adverse life events.

The majority of these models include manipulations that interrupt dam–pup interactions. In rats, during the first 20 days after birth (weaning), the dam's nurturing behavior (such as licking, grooming, and feeding) is necessary for the normal development of many systems in the pup, such as the nervous system and the stress response system (3,4). Interrupting dam–pup interactions during this period may cause severe developmental abnormalities in the pups. Early adverse life event models in rats are designed to affect these interactions. The long-term effects of these events typically depend on the duration of these early manipulations. Generally speaking, long-term separation of the dam from the pups (3–6 h/day) causes developmental abnormalities of the nervous system (5–9) and the stress response system (4,10–12), as well as an increase in behaviors reflecting anxiety (13–15), depression (16–22), schizophrenia (6,23–27), and substance dependence (28–32) in adulthood. In contrast, short-term separation of the dam from the pups (15 min/day) may exert positive effects on neurodevelopment (5,7), cause a less sensitive stress response system (33,34), and result in fewer behaviors reflecting anxiety (34–37) and depression (16,18,20–22) in adult rats, which is referred to as 'toughening-up' (4).

To our knowledge, the effect of an ambivalent mother as an early adverse life event has yet to be studied in an animal model. Being raised by an ambivalent mother has been described as a risk factor for developing many

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psychiatric disorders, such as schizophrenia, in humans (38–40). In this study, we aimed to create an "ambivalent mother" model in rats and to study the behavioral and neurodevelopmental effects of this model. Our hypothesis was that the effects of an ambivalent mother would be as strong as severe early adverse life events. For this purpose, newborn rats were randomly placed into four groups according to the postnatal manipulations they received: 1) animal facility rearing (AFR), 2) early handling (EH), 3) early deprivation (ED), and 4) ambivalent mother (AM). When they became adults, their behaviors and neurodevelopment levels were analyzed.

2. Materials and methods

2.1. Dams and subjects

This study was performed in the Neuroscience Laboratory of Gazi University. Ethical approval was obtained from the Gazi University Animal Studies Ethics Committee. Eight pregnant female Wistar rats and 72 pups born of these mothers were included in this study. The distribution of the pups among mothers and the entire study design are presented in Table 1.

The mothers were kept in a controlled environment in which the room temperature was 22 ± 2 °C and the humidity was 55 ± 5 %. The dark/light cycle was 12 h of daylight and 12 h of darkness. The mothers received food and water ad libitum and were transferred to a high-protein pellet diet during lactation. Examination of the mothers for the presence of pups was performed twice daily, at 0900 and 1700 hours.

2.2. Postnatal manipulations

The mothers and the pups were randomly separated into four postnatal treatment groups: 1) AFR, 2) EH, 3) ED, and 4) AM.

The AFR group was disturbed only by animal facility practices (e.g., removal of the dams and the pups for a few minutes for cage cleaning 2–3 times a week) and was otherwise left undisturbed. The procedures for EH, ED, and AM were performed during postnatal days (PNDs) 8–21 at 0900–1200 hours.

EH included the removal of the dam from the home cage, the removal of the pups from the home cage, and the placement of the pups individually in a cage containing compartments (10 × 10 × 20 cm), which was placed on a heating table (28–30 °C). After 15 min, the pups and subsequently the dam were returned to the home cage (4,41,42).

For ED, initially, a previously described procedure was used (42–45). In this procedure, on PND 1, the dam and subsequently the pups were removed from the home cage, and the pups were placed individually in the cage described above. After 3 h of separation, the pups and subsequently the dam were returned to the home cage. However, because at PND 7, 90% of the litters had died, it was necessary to modify this classic model. Given that the possible reasons for these deaths were hypoglycemia and/or hypothermia, the following modifications were made:

- All postnatal procedures were performed on PNDs 8–21 (instead of PNDs 1–14) because both the glycemic and thermal regulation systems of the pups were developed at that time.
- To protect against hypoglycemia, the pups were fed 1 mL of sucrose after 1.5 h of separation. For this procedure, the pups were handled briefly, and 1 mL of sucrose was placed in their mouths with an insulin injector.
- To protect against hypothermia, in addition to placing the cage on a heating table (28–30 °C), the temperature of the room was maintained at 30–32 °C during the separation period. The body temperature of the pups was recorded daily after 1.5 h of separation. For this procedure, one of the pups was chosen randomly to be briefly handled, and a thermometer was placed in its mouth for a few seconds. The body temperature of the pups was never detected to be below 35 °C.

To our knowledge, this the first study to conduct an ambivalent mother model using rats. In this model, a plush toy rat was used as a "fake mother". This fake mother resembled an adult rat and smelled similar to the real mother because it was placed in the same cage as the real mother for 30 min/day during the final 3–4 days of pregnancy. However, because it was a toy, it did not provide any maternal behaviors such as carrying, licking, grooming, or nursing. For the AM model, during PNDs 8–21, the real mother was removed from the home cage, and the fake mother was placed in the home cage for 3 h/day. The same precautions for hypoglycemia and hypothermia described above were also included in this procedure. We considered that this model represented an ambivalent mother in humans because the pups were exposed to opposing maternal behaviors: sometimes the mother was loving and caring (the real mother), but at other times the mother was cold and disinterested (the fake mother). We observed that even if we placed the fake mother in a remote area of the cage, the pups would quickly approach this fake mother, trying to get her attention and/or exhibiting caring behaviors (e.g., they would lick and touch the mother, and they would move under the mother). Furthermore, we think that this behavior cannot be explained only by an interest in a novel object because the pups continued to approach this mother every day during this period. Images of the fake mother and the interactions between this mother and pups are shown in Figure 1.

Weaning was performed on PND 21. The male and female litters were placed in different cages to prevent any

Figure 1. Photos of the "fake mother" and the interaction between this mother and the pups.

pregnancies. All animals received standard care during PNDs 22–60. As shown in Table 1, during PNDs 61–88, behavioral tests were performed as described below.

2.3. Behavioral analysis

On PND 62, the behaviors of the rats were automatically recorded using a noninvasive behavioral analysis system. This system is composed of a standard rat cage fixed to a platform containing several force-displacement transducers that is connected to a personal computer (Laboras, Metris, the Netherlands). The platform detects and classifies the behaviors according to the vibrations generated by the movement of the animals (46–48). Behaviors such as freezing, grooming, drinking, eating, locomotion, immobility, and rearing were recorded for 15 min. All experiments were simultaneously recorded using a video camera system to confirm the data obtained from the automated analysis system and to differentiate freezing from immobility.

2.4. Ultrasonic vocalization recordings

Many vertebrates use species-specific vocalizations to communicate information regarding mother–offspring interactions, mating, mood (fear, pain, distress, aggression, joy, etc.), their next planned behavior (approaching, avoidance, and grooming), and environmental conditions (presence of predators or the location of food). This information is important for understanding the behaviors of animals under laboratory conditions (49).

Adult rats primarily emit two types of ultrasonic vocalizations (USVs) that are distinguished based on the frequency displaying the peak amplitude. The vocalizations typically referred to as 22-kHz vocalizations occur at frequencies between 18 and 32 kHz for a duration of 300 to 4000 ms at a sound pressure level of 65 to 85 dB (50). Rats emit 22-kHz vocalizations in response to several aversive behavioral situations during distressing events, and it is assumed that these vocalizations reflect a negative affective state of the animal (for a review, see the work of Portfors (49)). The 50-kHz vocalizations occur at a frequency displaying a peak amplitude of 32 to 96 kHz, and these vocalizations occur for a much shorter duration (from 30 to 50 ms). Occasionally, 50-kHz vocalizations are referred to as chirps because of their brief duration (51). Rats emit 50-kHz vocalizations under nonaversive conditions, including sexual behavior, play, and manual tactile stimulation (tickling), and it has been suggested that these vocalizations are associated with the positive effect of the animal (for a review, see the work of Portfors (49)).

These vocalizations are inaudible to humans without the use of specialized equipment. Ultrasonic sounds

Table 1. Design of the study.

	Control	Early handling	Early deprivation	Ambivalent mother
Dams and pups	Mother A: 8 pups Mother B: 6 pups	Mother C: 12 pups Mother D: 8 pups	Mother E: 6 pups Mother F: 9 pups	Mother G: 14 pups Mother H: 9 pups
Postnatal days				
(PNDs) 1–7	Standard care			
PNDs 8–21	Standard treatment	Early-handling model	Early-deprivation model	Ambivalent mother model
PNDs 22–30	Standard care			
PND 31	Separation of pups from the mothers and of male/female siblings			
Sex distribution	6 males 8 females	11 males 9 females	5 males 10 females	7 males 16 females
PNDs 32–60	Standard care			
PND 61- Sex distribution	6 males 6 females	10 males 6 females	4 males 9 females	4 males 13 females
Day (D) 62	Behavioral analysis and ultrasonic vocalization recordings			
D 63–68	Standard care			
D 69	Elevated plus maze			
D 70–76	Standard treatment			
D 77	Forced swim test- pretest session			
D 78	Forced swim test- test session			
D 79–84	Standard care			
D 85–86	New object recognition test- habituation session			
D 87	New object recognition test- short-term memory session			
D 88	New object recognition test- long-term memory session Removal of brain tissue			

(within the range of 15–100 kHz) produced by laboratory animals are monitored and analyzed using a USV detector system (Sonotrack, Metris, the Netherlands). The Sonotrack contains a hardware bandpass filter (10th order Butterworth filter) using sharp thresholds at 15 kHz and 100 kHz. This filter prevents aliasing and also removes almost all environmental sounds. The data are presented without further filtering or smoothing. In the Sonotrack, the dB scale is relative to a 1-mV (RMS) signal. In the spectrogram, the color red indicates the strongest signal value (50 mV in a Sonotrack, 35.3 V RMS, or 31 dB), and black indicates the background noise (approximately 10 mV in a Sonotrack, 7 mV RMS, or 16 dB). This shift in the frequency at the beginning and at the end of the USV is characteristic of a biological sound.

In this study, we used a Sonotrack to detect the USVs of all animals during day 0 and day 14 of the behavioral analysis, as well as during the pretest and test sessions of the forced swim test (FST). The detected sounds were then grouped into either Band I (vocalizations of 18–32 kHz, related to distress) or Band II (vocalizations of 32–50 kHz, related to a positive effect).

2.5. Elevated plus maze (EPM)

The EPM has been described as a simple method of assessing the anxiety responses of rodents by Pellow et al. (52). The EPM used in this study was constructed of Plexiglas and consisted of two open arms (50 cm long, 10 cm wide) and two closed arms (50 cm long, 10 cm wide, enclosed by 30 cm walls). Each arm was attached to plastic legs, such that it was elevated 50 cm above the floor. The animals were placed individually in the center of the maze facing the same closed arm and were allowed 5 min of free exploration. A video camera was placed above the EPM to simultaneously record the behavior of the rats, and an

observer recorded the number of entries into each arm and the time spent in each arm. The maze was thoroughly cleaned after each test using alcohol. Each rat was tested once. The final results were calculated as percentages (53):

$$\text{Percentage of closed (open) arm entries} = \frac{\text{Number of closed (open) arm entries}}{\text{Number of total arm entries}} \times 100$$

$$\text{Percentage of duration spent in closed (open) arm (seconds)} = \frac{\text{Mean duration spent in closed (open) arm}}{\text{Total duration spent in any arm}} \times 100$$

2.6. Forced swim test (FST)

The FST, as originally reported by Porsolt et al. (54), has become the most widely used model for assessing antidepressant-like activity in rodents. For this test, a glass cylinder with a depth of 60 cm and a diameter of 30 cm was used. The cylinder was filled with 30 cm of tap water at 23–25 °C. For the pretest (habituation) session, the rats were placed individually into the cylinder, allowed to swim for 15 min, removed from the water, dried under a lamp, and returned to their home cage. For the test session, 24 h later, the same procedure was performed, but during this session, the duration in the cylinder was 5 min. During the test session, an observer recorded the total time the rats spent performing three different types of behaviors: (1) climbing, defined as upward-directed movement of the forepaws, typically against the side of the swim cylinder; (2) swimming, defined as the horizontal movement of the rat within the cylinder, and (3) immobility, defined as floating in the water without struggling and only making the necessary movements to maintain its head above water (55). All sessions were simultaneously recorded using a video camera system, and the USVs were recorded using a Sonotrack.

2.7. Novel object recognition (NOR) test

The NOR test is used to evaluate short- and long-term recognition memory in mice and rats (56,57). For this test, an open field and three groups of objects (Lego toys similar in texture and size but different in shape and color) were used. The weight of the objects ensured that they could not be displaced by the rats. The NOR test consisted of four stages: 1) habituation- the subjects were placed in the open field for 10 min/day for two consecutive days, in which no objects were present; 2) training session- 24 h after the second habituation session, the rats were placed individually in the open field for 10 min, in which two identical objects (A1 and A2) were placed symmetrically in two adjacent corners 10 cm from the walls; 3) short-term memory session- 1 h after the training session, the rats explored the open field for 5 min in the presence of one familiar (A3) and one novel (B1) object; and (4) long-term memory session- 24 h after the training session, the rats were placed in the open field for 5 min in the presence of one familiar (A4) and one novel (C1) object. The same objects were used for every rat, and the objects and the open field were cleaned using a 95% ethanol solution between trials. An animal was considered to be exploring the object when it directed its nose toward the object at a distance of less than 2 cm from the object, including touching the object. Object exploration was measured using two stopwatches to record the time spent exploring the objects during the short- and long-term memory sessions by an investigator blinded to the treatment group of the animals. The animals were videotaped during all sessions. Two indexes were calculated for both short- and long-term memory (58) (T_N, the time spent exploring the novel object, and T_F, the time spent exploring the familiar object):

$$\text{Recognition index (RI)} = \frac{T_N}{T_N + T_F}$$

$$\text{Discrimination index (DI)} = \frac{T_N - T_F}{T_N + T_F}$$

2.8. ELISA

2.8.1. Sample preparation

Two hours after the long-term memory session of the NOR test, the rats were decapitated after an intraperitoneal injection of thiopental sodium (30 mg/kg). The brains were removed and the hippocampus, the prefrontal cortex, and the cerebellum were dissected according to Rat Brain Atlas coordinates. These tissues were placed in Eppendorf tubes, immersed immediately in liquid N_2, and stored at –80 °C until ELISA. The samples were homogenized in a radioimmunoprecipitation assay buffer supplemented with a 2% protease inhibitor cocktail. The homogenates were centrifuged at 14,000 × *g* at 4 °C for 30 min.

2.8.2. ELISA procedure

The total brain-derived growth factor (BDNF) protein levels were determined via a sandwich enzyme immunoassay using a commercial ELISA kit (ChemiKine, Cat. No. CYT306, Merck Millipore). The measurements were performed according to the manufacturer's instructions. The limit of sensitivity was set at 7.8 pg/mL. The intraassay variability was 3.7% and the interassay variability was 8.5%. All assays were performed in duplicate. The BDNF levels were expressed as pg/mL.

2.9. Statistical analysis

Statistical analysis was performed using SPSS 15.0. Nonparametric tests were used. For between-group

comparisons, the Kruskal–Wallis test was performed. For within-group comparisons, the Wilcoxon test was performed. The Mann–Whitney U test was performed to determine which groups were significantly different.

3. Results

The study design is shown in Table 1. There was no significant difference between the weights of the groups, but the EH group contained a significantly greater number of male rats (n = 10, 62.5%) than the AM group (n = 4, 23.5%, $\chi^2 = 0.105$, P = 0.044).

3.1. Behavioral analysis

A comparison of the behaviors of the groups is shown in Figure 2. In general, there was a significant difference between the groups in the mean duration (seconds) of head grooming ($\chi^2 = 11.550$, P < 0.01), body grooming ($\chi^2 = 13.200$, P < 0.01), drinking behavior ($\chi^2 = 10.525$, P < 0.01), locomotor activity ($\chi^2 = 20.304$, P < 0.01), and rearing ($\chi^2 = 16.089$, P < 0.01). These significant differences were caused by the female rats but not by the male rats. The female rats in the ED and AM groups spent significantly less time head grooming (mean ± standard error: 21.78 ± 5.70 and 37.31 ± 7.19), body grooming (9.44 ± 2.44 and 13.62 ± 4.10), and rearing (512.56 ± 17.14 and 490.31 ± 18.69) than those in the control group (89.33 ± 16.13, 140.00 ± 38.84, and 312.00 ± 28.73, respectively) and EH group (105.00 ± 11.47, 69.00 ± 20.61, and 364.50 ± 40.42, respectively; for head grooming, $\chi^2 = 19.562$, P < 0.01; for body grooming, $\chi^2 = 19.397$, P < 0.01; and for rearing, $\chi^2 = 19.510$, P < 0.01). The female rats in the AM group (103.08 ± 7.92) exhibited significantly higher locomotor activity than those in the control group (58.83 ± 9.81) and EH group (49.00 ± 8.03; $\chi^2 = 16.955$, P < 0.01).

3.2. Elevated plus maze (EPM) test

The performance of the groups in the EPM test is presented in Figure 3. According to these results, there was a significant difference between the groups in the percentage of open arm entries ($\chi^2 = 11.709$, P < 0.01) and the percentage of time spent in the open arms ($\chi^2 = 17.900$, P < 0.01). The ED (16.46 ± 5.29) and AM (10.08 ± 3.11) groups displayed a significantly higher percentage of time spent in the open arms than the control (2.48 ± 1.02) and EH (0.92 ± 0.68) groups. The ED (19.24 ± 3.71) and AM (16.49 ± 3.91) groups exhibited a significantly higher percentage of open arm entries than the EH group (3.48 ± 1.66). With respect to the percentage of duration time in the open arms, the female rats, but not the male rats, exhibited this significant difference. The female rats in the ED (16.64 ± 5.92) and AM (12.22 ± 3.88) groups exhibited a significantly higher percentage of time spent in the open arms than those in the control group (2.99 ± 1.69) and EH group (0.38 ± 0.25; $\chi^2 = 11.186$, P = 0.011).

3.3. Forced swim test (FST)

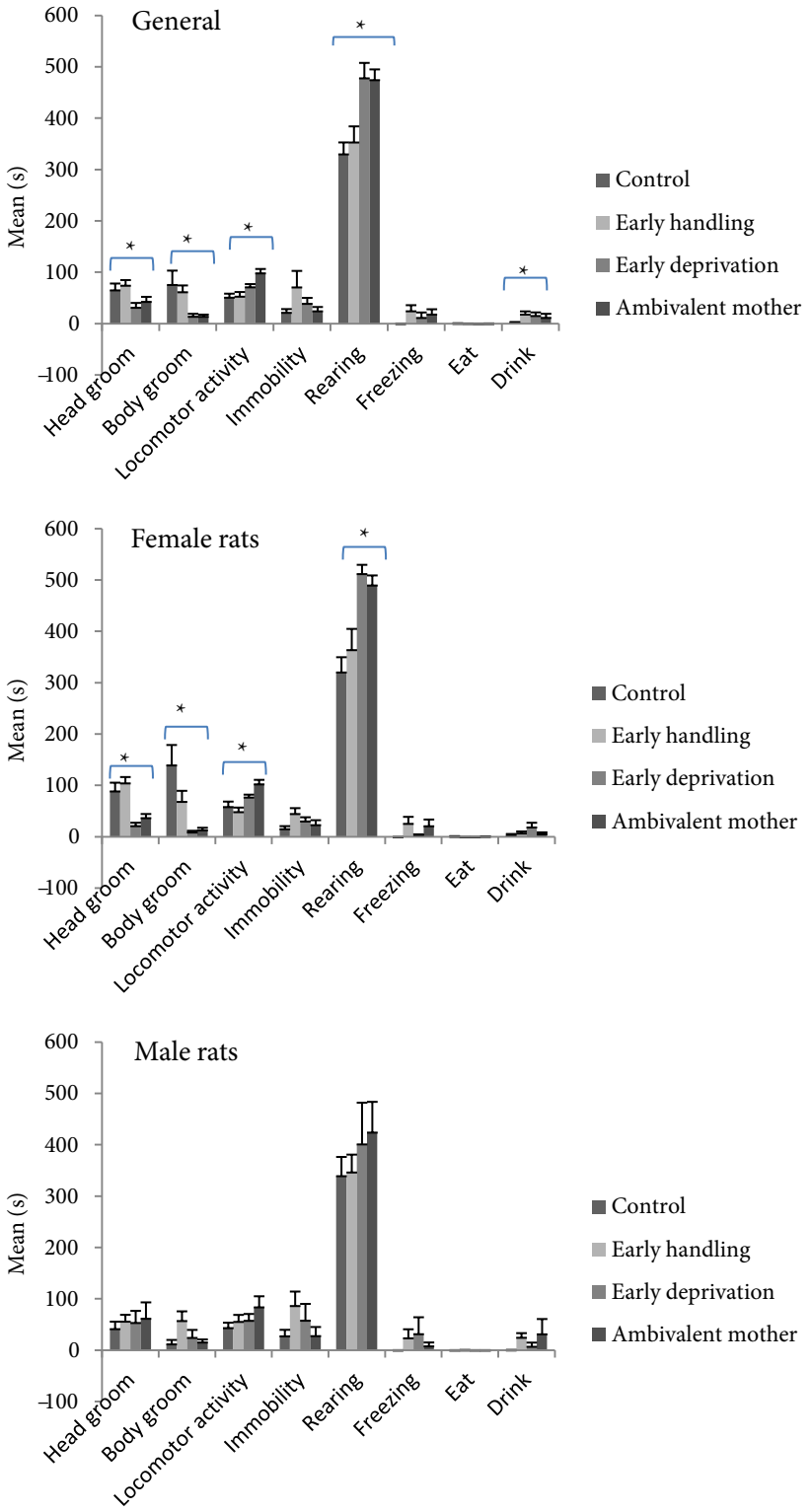
The FST results are shown in Figure 4. There was a significant difference between the groups in the mean duration of climbing ($\chi^2 = 16.308$, P < 0.01), swimming ($\chi^2 = 15.170$, P < 0.01), and immobility ($\chi^2 = 26.832$, P < 0.01). The EH group spent significantly less time climbing (19.75 ± 4.63) than the control (39.83 ± 8.42), ED (47.00 ± 10.03) and AM (63.35 ± 7.26) groups. The ED (77.00 ± 13.54) and AM (85.35 ± 7.98) groups spent significantly more time swimming and significantly less time immobile (176.77 ± 14.23 and 145.53 ± 8.50, respectively) than the control (38.08 ± 5.17 and 230.42 ± 10.23, respectively) and EH (53.06 ± 10.59 and 227.19 ± 10.82, respectively) groups. For the male rats, the AM group (164.25 ± 13.65) exhibited a significantly shorter mean duration of immobility than the control group (229.83 ± 7.56) and EH group (240.50 ± 11.07; $\chi^2 = 7.866$, P = 0.049). For the female rats, the ED and AM groups spent significantly more time swimming (86.00 ± 12.68 and 92.38 ± 8.77, respectively; $\chi^2 = 11.413$, P = 0.010) and significantly less time immobile (160.44 ± 11.48 and 139.77 ± 10.00, respectively; $\chi^2 = 15.347$, P < 0.01) than the control group (34.67 ± 8.46 and 231.00 ± 20.08, respectively) and EH group (65.67 ± 26.24 and 205.00 ± 20.28, respectively).

3.4. New object recognition (NOR) test

The results of the NOR test are shown in Tables 2 and 3. There was no statistically significant difference between the four groups in either the discrimination or recognition index for short- or long-term memory, even when the rats were analyzed according to their sex. However, interestingly, there were statistically significant differences between the groups in the duration spent with the familiar and novel objects.

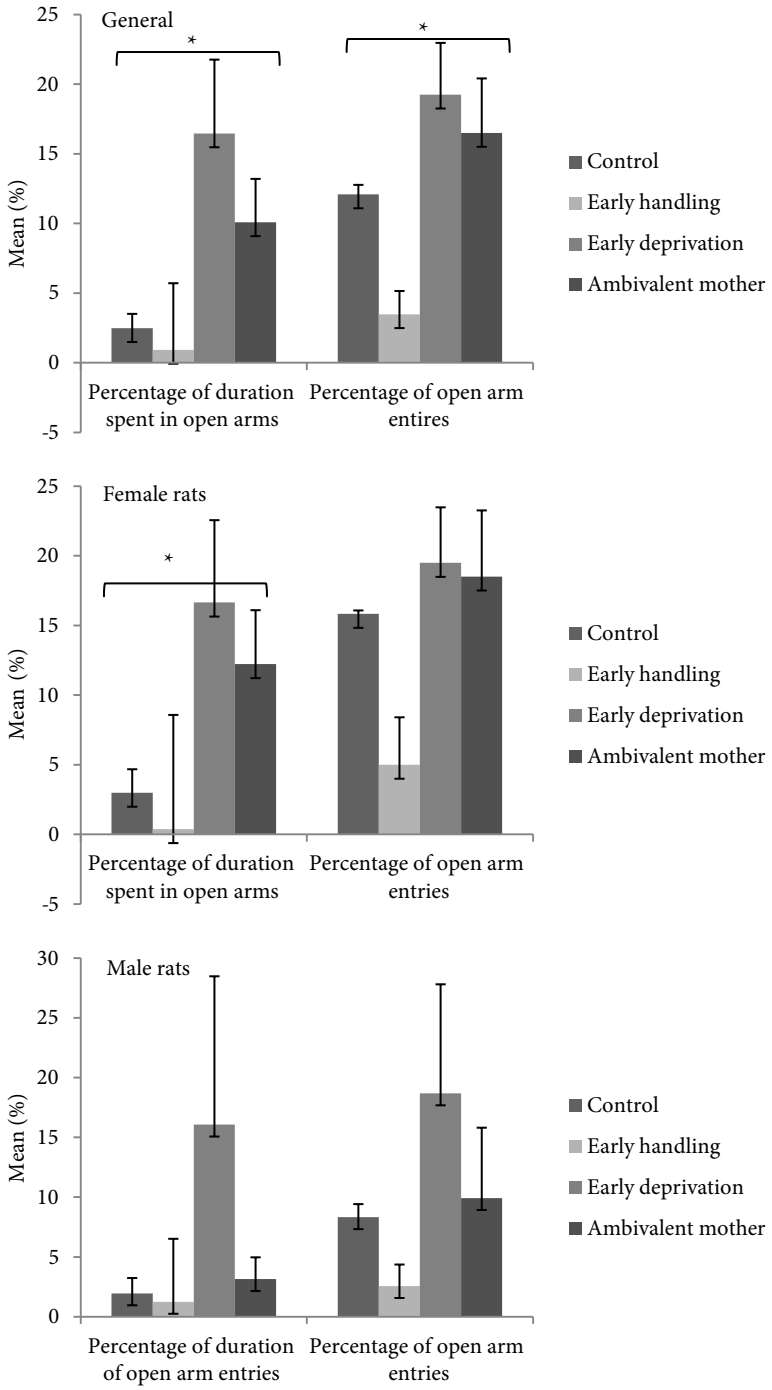
During the short-term memory session of the NOR test, the ED (10.23 ± 1.84) and AM (13.35 ± 3.71) groups spent significantly more time with the familiar object than the control group (3.00 ± 0.73) and EH group (4.00 ± 2.57; $\chi^2 = 15.564$, P < 0.01). The AM group (23.85 ± 5.37) spent significantly more time with the novel object than the control group (6.00 ± 1.86) and EH group (10.00 ± 3.76; $\chi^2 = 10.706$, P = 0.013). The ED (34.08 ± 6.64) and AM (29.06 ± 6.71) groups spent significantly more total time with either object than the control group (9.00 ± 2.19) and EH group (14.10 ± 4.13; $\chi^2 = 14.654$, P < 0.01). These results were similar for both sexes (data not shown).

During the long-term memory session of the NOR test, the ED (10.00 ± 1.45) and AM (10.47 ± 2.13) groups spent significantly more time with the familiar object than the control group (4.92 ± 1.75) and EH group (4.69 ± 0.83; $\chi^2 = 11.668$, P < 0.01). The AM group (13.85 ± 2.21) spent significantly more time with the novel object than the control group (4.25 ± 1.76) and EH group (8.31 ± 2.46; $\chi^2 = 10.451$, P = 0.015). The ED (23.84 ± 2.83) and AM (21.00 ±



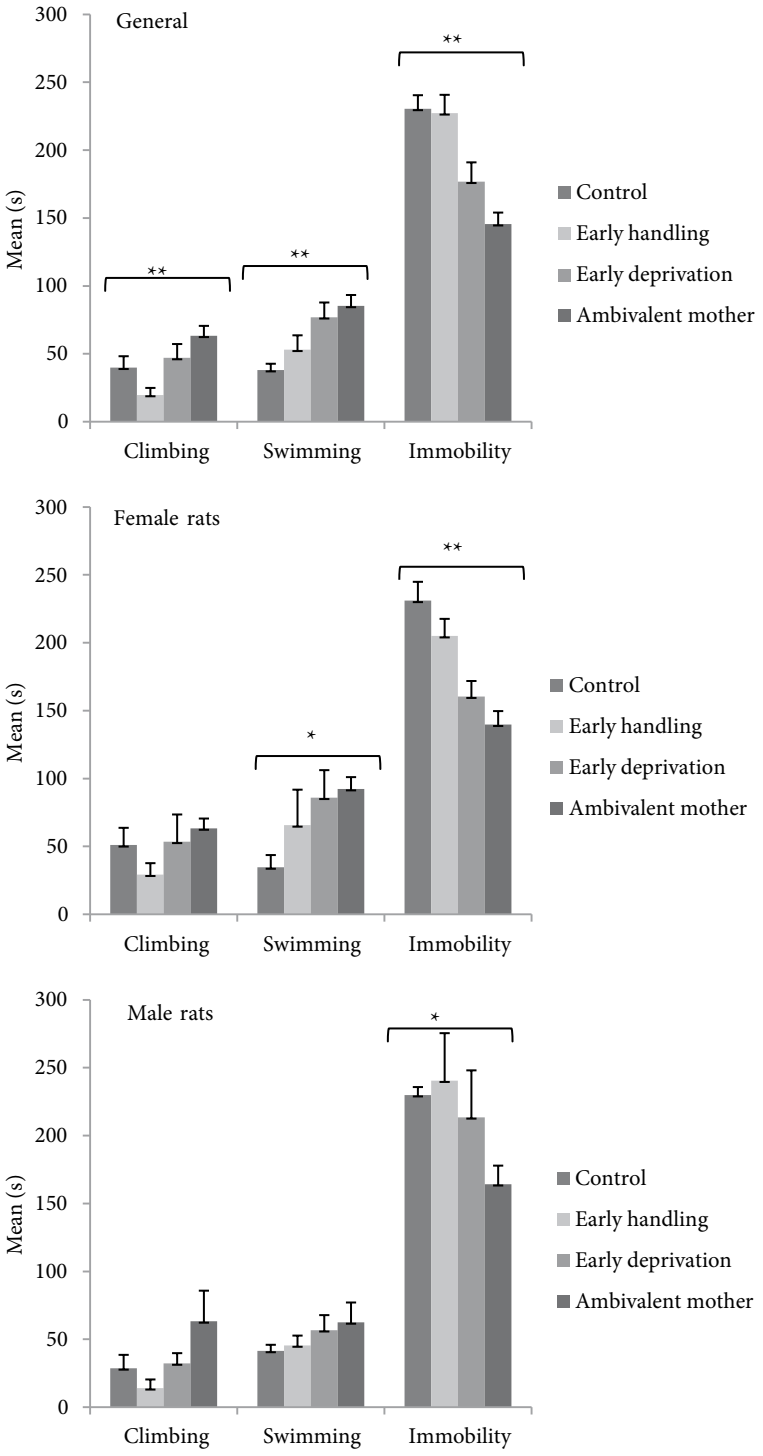
*: p<0.01

Figure 2. Behavioral analysis of the groups.



*: $p<0.01$

Figure 3. Results of the elevated plus maze.



*: $p<0.05$, **: $p<0.01$

Figure 4. Results of the forced swim test.

Table 2. Results of short-term memory sessions of the NOR test.

	Control	Early handling	Early deprivation	Ambivalent mother	χ^2	P
	Mean (s) ± standard error					
General						
RI	0.47 ± 0.02	0.59 ± 0.02	0.66 ± 0.02	0.47 ± 0.02	4.052	0.256
DI	0.01 ± 0.17	0.16 ± 0.12	0.27 ± 0.02	0.01 ± 0.10	1.760	0.624
Males						
RI	0.61 ± 0.02	0.56 ± 0.02	0.66 ± 0.02	0.48 ± 0.10	2.644	0.450
DI	0.23 ± 0.19	0.80 ± 0.11	0.19 ± 0.16	–0.01 ± 0.20	1.179	0.758
Females						
RI	0.34 ± 0.15	0.64 ± 0.13	0.66 ± 0.02	0.47 ± 0.02	4.589	0.204
DI	–0.20 ± 0.26	0.28 ± 0.27	0.31 ± 0.12	0.09 ± 0.12	3.559	0.313

RI: Recognition index, DI: discrimination index.

Table 3. Results of long-term memory sessions of the NOR test.

	Control	Early handling	Early deprivation	Ambivalent mother	χ^2	P
	Mean (s) ± standard error					
General						
RI	0.42 ± 0.10	0.49 ± 0.02	0.57 ± 0.02	0.39 ± 0.02	3.102	0.376
DI	0.24 ± 0.17	0.01 ± 0.15	0.01 ± 0.10	0.17 ± 0.11	2.251	0.522
Males						
RI	0.38 ± 0.16	0.52 ± 0.10	0.55 ± 0.12	0.47 ± 0.13	1.286	0.733
DI	–0.06 ± 0.28	0.04 ± 0.21	0.11 ± 0.24	0.41 ± 0.14	2.099	0.552
Females						
RI	0.46 ± 0.14	0.44 ± 0.02	0.58 ± 0.02	0.37 ± 0.02	3.175	0.365
DI	0.10 ± 0.23	–0.05 ± 0.20	–0.04 ± 0.11	0.10 ± 0.14	1.873	0.599

RI: Recognition index, DI: discrimination index.

4.27) groups spent significantly more total time with either object than the control group (9.16 ± 3.26) and EH group (13.00 ± 3.06; χ^2 = 12.303, P < 0.01). These results were similar for both sexes (data not shown).

3.5. Ultrasonic vocalizations (USVs)

No USVs were detected during the behavioral analysis, the EPM test, or the test session of the FST. During the pretest session of the FST, one animal from the control group (8.3%) performed several 22-kHz vocalizations (during the first 2 min) and several 50-kHz vocalizations (after 7 min), and one animal from the AM group (5.9%) performed two 50-kHz vocalizations (at 14 min; χ^2 = 2.198, P = 0.532).

3.6. ELISA

The BDNF protein levels in the prefrontal cortex (χ^2 = 4.857, P = 0.302), the dentate gyrus (χ^2 = 3.526, P = 0.317), and the cerebellum (χ^2 = 4.857, P = 0.302) of the four groups did not display any significant differences.

4. Discussion

The results of the behavioral analysis performed using Laboras and direct observation revealed that the ED and AM groups exhibited similar behaviors that were significantly different from those of the control and EH groups. The ED and AM groups exhibited significantly

less grooming behavior (both head and body) and were significantly more hyperactive (as measured by locomotor activity and rearing) than the control and EH groups. These significant differences were attributed to the females rather than the males. It is well known that grooming behavior is an indirect indicator of high levels of stress and pain in rodents (59–62). It has been proposed that the increased frequency of grooming behavior reflects the behavioral profile of rodents exposed to anxiety-provoking stimuli (59). Locomotor activity and rearing behavior in a novel environment are related to inquiring behavior, and higher levels of these activities reflect a lower level of anxiety (63–66). Thus, based on the results of our behavioral analysis, we conclude that the female rats in the ED and AM groups were less anxious than those in the control and EH groups.

The finding that the ED and AM groups were less anxious than the control and EH groups, observed by behavioral analysis, is further supported by our findings in the EPM test. According to the EPM results, the rats in the ED and AM groups were significantly less anxious that those in the control and EH groups, as measured by the more frequent open arm entries and longer times spent in the open arms in the EPM. Again, this significance was attributed to the female rats rather than the male rats. In a review of the effects of early postnatal manipulations on anxiety models in rodents, it was determined that the EH model decreases the anxiety level in adulthood, whereas longer dam–pup separations during the early postnatal period (such as ED) increase anxiety in adulthood (19). However, there is conflicting evidence regarding the effects of early adverse events on the performance of adult rats on the EPM test in the previous literature. Most previous studies reported a significant decrease in anxiety levels in the EH model (34,35,37). Longer dam–pup separation has been associated with higher levels of anxiety (11,13–15,20). However, in our study, we found the opposite results. This may be due to our modifications of the procedures for the ED and AM models, which are discussed below.

Our results regarding depressive behavior are similar to those regarding anxiety levels: the ED and AM groups exhibited significantly less depressive behavior, as demonstrated by the reduced time immobile during the FST compared to the control and EH groups for both the female and male rats. In general, previous studies reported that postnatal manipulations that cause longer dam–pup separation increase depressive behavior (such as a decrease in sucrose intake, sleep disturbances, lack of appetite, etc.) in rats (17,19). However, the results of the FST are inconclusive. Some previous studies reported an increase in immobility during the FST after longer dam–pup separations (such as ED) compared to normally raised rats and rats that were exposed to shorter dam–pup separations (such as EH) (16,18,20–22,43). The contrast between our results and those of previous reports may be due to our modifications of the procedures for the ED and AM models, which are discussed below.

With regard to memory function, which was evaluated using the NOR test in this study, we did not detect any significant difference between the groups. However, the rats in the ED and AM groups exhibited significantly more interest in both objects than those in the control and EH groups. Most previous studies reported that shorter dam–pup separations exert a positive effect on cognitive functions in adulthood (67–70), whereas longer dam–pup separations exert a negative effect (5,16,71,72). The same is true for neurodevelopment, as measured by the BDNF protein levels in the brain in this study.

In summary, our results demonstrate that longer dam–pup separation (the ED and AM models) during the early postnatal period decrease adulthood anxiety levels in females and decrease adulthood depressive behavior in both sexes. This finding is in contrast to our hypothesis and most previous findings. We think that this contradiction may be due to our modifications of the procedures for the ED and AM models. As explained in Section 2, we had to take precautions to prevent hypoglycemia and hypothermia, which were the possible causes of death while applying the classical ED model. The first precaution was to apply the model during PNDs 8–21 (instead of the classical PNDs 1–14) because both the glycemic and thermal regulation systems of the pups are more developed at that time. By taking this precaution, we may have missed the "critical" period of neural and stress response system development, which occurs on PNDs 3–14 (4,12). The second precaution was to feed the pups 1.5 mL of sucrose every day after 1.5 h of separation to protect them against hypoglycemia. Thus, we had to handle the pups for a brief period daily. Therefore, these pups received more tactile stimulation than those in the control and EH groups. It was previously reported that neonatal tactile stimulation may alleviate the negative effects of early adverse events in rats (73–76). The third precaution was to apply the model in a warmer environment (room temperature of 30–32 °C) than the classic model and to measure the body temperature daily after 1.5 h of separation. It was previously reported that applying the ED model during daytime and at 32 °C (ED- light and warm) may cause 'toughening-up' (4,77). Moreover, handling the pups daily to measure their body temperature resulted in additional neonatal tactile stimulation.

Thus, these modifications may be responsible for our findings that longer dam–pup separations caused 'toughening-up' and decreased anxiety and depressive-like behaviors in adulthood. However, one may argue that these results can solely be explained by a decrease in anxiety and depression. Our personal observation was that the ED and

AM groups were "more active" and "more aroused" than the control group. This observation is further supported by our findings that the ED and AM groups exhibited significantly more locomotor activity and rearing during behavioral analysis, entered more arms during the EPM test, performed more swimming and climbing during the FST, and were more interested in the objects during the NOR test. It has been proposed that hyperactivity and hyperarousal may occur in rodent models of psychosis and mania (78–81). Because we did not use a specific tool to evaluate the psychosis and mania of our sample, we cannot exclude this possibility. Investigating the effects of the AM model on adulthood psychosis and mania may be an interesting area of investigation in the future.

In addition to the limitations caused by these modifications, another important limitation is that there were significant sex differences between our groups. The EH group contained significantly fewer female rats than the AM group. However, the EH group exhibited significantly more anxiety-like behavior than the AM group. Sex may exert an effect on the behavioral and neurobiological consequences of adverse life events, but it is impossible to clearly identify a sex effect because the results are in conflict with one other (6,8,10,11,14,22).

Despite these limitations, we think that our study is of interest because it is the first to use an ambivalent mother model as an early adverse event in rats. Future studies are clearly needed to determine the reliability and specificity of this model. We think that applying the ambivalent mother model during PNDs 1–14 and without frequent neonatal tactile stimulation is necessary to better understand the effects of being raised by an ambivalent mother on rats.

Acknowledgment

This work was supported by a grant (01/2011-60) from the Scientific Research Project Department of Gazi University.

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