Research Article / *Araştırma Makalesi*

DOI: 10.4274/npa.y5700

Effects of Early Litter Loss on Maternal Behavior and Subsequent Generations in Rats: An Attempt for an Animal Model for Loss of a Baby

Erken Yavru Kayıplarının Sıçanlardaki Annelik Davranışına ve Sonraki Yavrulara Etkileri: Bebek Kaybının Hayvan Çalışması Modeli

Aylin ÖZBEK, Mustafa AKHİSAROĞLU*, Onur Burak DURSUN, Fatma VAROL TAŞ, Osman YILMAZ**, İlgi ŞEMİN*

Dokuz Eylül Üniversitesi Tıp Fakültesi, Çocuk ve Ergen Ruh Sağlığı ve Hastalıkları Anabilim Dalı, İzmir, Türkiye

*Dokuz Eylül Üniversitesi Tıp Fakültesi, Fizyoloji Anabilim Dalı, İzmir, Türkiye

**Dokuz Eylül Üniversitesi Tıp Fakültesi, Deney Hayvanları ve Deney Hayvanlarının Sağlık Bilimlerinde Kullanımı Anabilim Dalı, İzmir, Türkiye

ABSTRACT

Objective: Loss of a baby accompanied by complicated grief increases vulnerability to mental disease with adverse consequences both for the mother and the long-term well-being of the subsequent generations. This study presents an animal model and aims to study the reactions of female rats to the loss of their litters and the consequences for the next litter.

Methods: Primiparious Sprague-Dawley rats were recruited into three groups: of separation-reunion, complete separation and no-separation. Maternal behaviors following separation, reunion and the birth of the subsequent litter were observed. The blood corticosterone levels of the dams in response to separation and reunion were measured. Learning, activity, anxiety and blood corticosterone response to stress of the second litters were evaluated.

Results: Dams in the separation group spent significantly more time at the nest following reunion, displayed increased pup licking in their second litters compared to their first litters and did not achieve the increased duration of nursing in their second generation litters as observed for the control group. Dams' blood corticosterone levels were significantly influenced by separation and reunion. Pups born to mothers that have experienced an early loss of their previous litters had significantly impaired cognitive abilities compared to controls.

Conclusion: Further analysis of the process of loss in animal models may have clinical implications for bereaved mothers. *(Archives of Neuropsychiatry 2011; 48: 44-52)*

Key words: Complicated grief, litter loss, maternal behavior, blood corticosterone level, learning

ÖZET

Amaç: Bebek kaybı ile oluşan komplike yas, hem anne hem de sonraki kuşaklar için olumsuz sonuçları olan, ruhsal hastalıklar açısından incinebilirliği arttıran bir durumdur Bu çalışma, yavru kayıplarının dişi sıçanlara etkilerini ve bir sonraki jenerasyon için doğurabileceği sonuçları araştırmayı amaçlayan bir hayvan modeli çalışmasıdır.

Yöntemler: Primipar Sprague-Dawley sıçanları ayrılma- birleşme grubu, tam ayrılık grubu ve ayrılık yaşamayan kontrol grubu olarak gruplanmıştır. Ayrılmayı, birleşmeyi ve bir sonraki yavruların doğumunu izleyen süreçlerde annelik davranışları gözlenmiş, kan kortikosteron düzeyleri ölçülmüştür. Grupların ikinci kuşak yavruların öğrenmeleri, aktivite düzeyleri, anksiyete durumları ve psikojenik stresöre maruziyetin ardından kan kortikosteron düzeyleri incelenmiştir.

Bulgular: Yavrularından ayrılıp, tekrar birleşen sıçanların birleşmeyi takiben yuvada anlamlı olarak daha fazla zaman geçirdikleri ve ikinci kuşak yavrularını anlamlı düzeyde daha fazla yaladıkları görülmüştür. Bu grubun kontrol grubunda gözlenen ikinci kuşak yavruları emzirme süresindeki anlamlı artışı göstermediği saptanmıştır. Anne sıçanların kan kortikosteron düzeyleri yavrulardan ayrılma ve birleşme sonrası anlamlı olarak etkilenmiştir. Erken yavru kaybı yaşayan annelerin sonraki kuşak yavrularında kontrol grubuyla karşılaştırıldığında anlamlı düzeyde daha fazla bilişsel bozulma saptanmıştır.

Sonuç: Hayvan modellerinde kayıp süreçlerinin çalışılmaya devam edilmesinin yas sürecindeki anneler ve bebekleri için klinik değeri olabilir.*(Nöropsikiyatri Arşivi 2011; 48: 44-52)*

Anahtar kelimeler: Komplike yas, yavru kaybı, annelik davranışı, kan kortikosteron düzeyi, öğrenme

Introduction

Loss of a loved one is considered to be among the most painful stress that humans would have to endure. When complicated with accompanying maladaptive behavior such as yearning, searching, disbelief, loneliness, emptiness, numbness and avoidance, losses and bereavement have been found to increase vulnerability to mental disease leading this area to be a focus of interest and research in psychology and psychiatry (1).

Address for Correspondence/Yazışma Adresi: Dr. Aylin Özbek, Dokuz Eylül Üniversitesi Tıp Fakültesi, Çocuk ve Ergen Ruh Sağlığı ve Hastalıkları Anabilim Dalı, İzmir, Türkiye Gsm: +90 505 805 86 57 E-posta: aylinozbektr@yahoo.com **Received/Geliş tarihi:** 29.06.2010 **Accepted/Kabul tarihi:** 14.08.2010

In the last two decades, clinical studies have been pointing to another aspect of loss, the perinatal loss, as having consequences both for the mother and the long-term well-being of the subsequent generations. It has been documented that 50% of cases of perinatal loss is followed by another pregnancy within 12 months, during which significantly larger number of mothers were receiving the diagnosis of anxiety disorders and depression, especially during the third trimester with well-established long-lasting effects for the fetus (2-4).

Increased levels of maternal anxiety have shown to induce hypoxia, arterial hypotension, bradicardia and abnormal adrenal gland responses in the fetus as well as craniofacial abnormalities, cardiac defects and low birth weight. Some behavioral consequences such as hyperactivity and cognitive deficits have also been mentioned as related to maternal anxiety during pregnancy (5-9).

Animal model work has been very useful in providing data regarding the physiological responses underlying behavioral, emotional reactions to loss since studies on variety of mammalian infants have revealed reactions resembling human responses (10). In this respect, long-term effects of loss occurring in infancy and early childhood on development have been studied extensively in animal models since clinical studies provide evidence that vulnerability to adult stress and predisposition to certain mental health disorders are increased in individuals with histories of early loss and/or early maternal deprivation (10).

Data suggest that regulation of the stress response is the main way, by which prenatal stressors exert their effects on the fetus (11). Although still not very clearly established, increase in the blood pressure in the uterine artery, associated with diminished blood supply to the fetus and hyperactivation of the maternal hypothalamo-pituitary-adrenal axis (HPA) leading to increased exposition of the fetus to high plasma glucocorticoid and mineralocorticoid levels, constitutes one of the core mechanisms held responsible (6). Additionally, effects of prenatal stress on quality of maternal care have also been found to be associated with altered regulation of the HPA in the offspring (12,13).

Along with clinical evidence, data from animal models have shown that beside prenatal circumstances, postnatal environment and rearing experiences also play an important role in the emotional responses and development of the offspring. It is suggested that in rodents, as well as other mammals, variations in maternal care such as licking/grooming of pups have been observed to alter gene expression, physiology, and behavior of both male and female offspring, exerting long-term neurobehavioral consequences for the pups (14). Data from numerous research applying maternal separation procedure have shown that a sequence of behavioral and physiological changes occurs in the pups when they were separated from the dam. While some researchers point to beneficial effects of brief periodic maternal separation on cognitive development of the offspring, most underline the <adverse effects (15). Immediate initiation of ultrasonic vocalizations, marked inhibition of the growth hormone (GH) and of the enzyme ornithine decarboxylase (ODC), an important mediator of nucleic acid synthesis, have been observed. Infants could become less responsive to sensory stimuli, body temperature and heart rate were decreased and the adrenal cortex began to secrete extraordinarily high levels of the glucocorticoid stress hormone corticosterone as separation was prolonged. Growth processes and behavioral activity decreased. Maternal separation also caused an increase in REM sleep (16). It has been also shown that separation of rat pups from their mothers increases behavioral fearfulness and induces long-lasting changes in reactivity to a novel environment and morphine-induced sensitization and tolerance (17). It has been suggested that vulnerability to depression, hyperactivity, cognitive deficits, neurological and behavioral abnormalities are increased in the offspring with histories of adverse postnatal environmental and emotional circumstances. Moreover, the above-mentioned adversities were suggested to impose transgenerational effects through mediating the maternal behavior of the female rat (8,12,18-20).

Despite the numerous research studies addressing the reactions of the offspring to maternal separation/loss and the clinical evidence that mothers react strongly to the loss of their children having consequences for the subsequent generations, the issue of how the animals react to the loss of their offspring have been explored scarcely (21). In one of the few reports regarding this issue, Boccia et al. stated that repeated, long separations of rat mothers from their pups induced a depression-like state, which may account for the reductions in maternal behavior (20).

Research addressing the questions of 'How does loss experienced by female rat alter the maternal behavior and physiology?' and 'What might be the consequences for the offspring of being born to a mother who had experienced previous litter loss?' could have helped to further study the biology and the transgenerational effects of perinatal loss - an area of research, where it is very difficult to conduct long-term prospective studies in humans due to inherent difficulties.

This study aims to explore how an animal model system can be used to study the reactions of postparturient female rats to the loss of their litters and the consequences for the next generation born to mothers who have previously experienced an early litter loss, as well as to aid in narrowing the gap between the psychology of loss and the biological aspects that are central to its role as a risk factor for mental well-being.

Methods

The design of the study is summarized in Figure 1.

Subjects and Housing

The subjects of this study were 37 primiparous 90-110 day old Sprague-Dawley rats obtained from Experimental Animal Laboratory, Dokuz Eylul University, School of Medicine. From the 37 rats, 17 participated in behavioral testing and 20 entered blood corticosterone measurement. Second generation pups of the 17 female adult rats constituted the additional subjects of the study.

All of the dams and their pups were housed individually in clear Plexiglas cages (22x44x30). The animals were provided with wood shavings. Food and water were available ad libitum. The animals were maintained on a 12:12 hour light/dark cycle with lights on at 08:00 hours. The room temperature and humidity were maintained at 24ºC and 40-50%, respectively.

All experiments were performed in accordance with the guidelines provided by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Procedure

Procedure for the Assessment of Maternal Reactions to Loss

All of the subjects participating in the research were mated, and following the normal 21-day pregnancy, first generation pups were born. At the postnatal day (PND) 6, maternal behavior recordings were applied to the dams. The experiments were initiated on PND 6 since it is reported that on and after PND 6, mothers' responsiveness to the pups are not solely affected by the hormones of parturition and the maternal

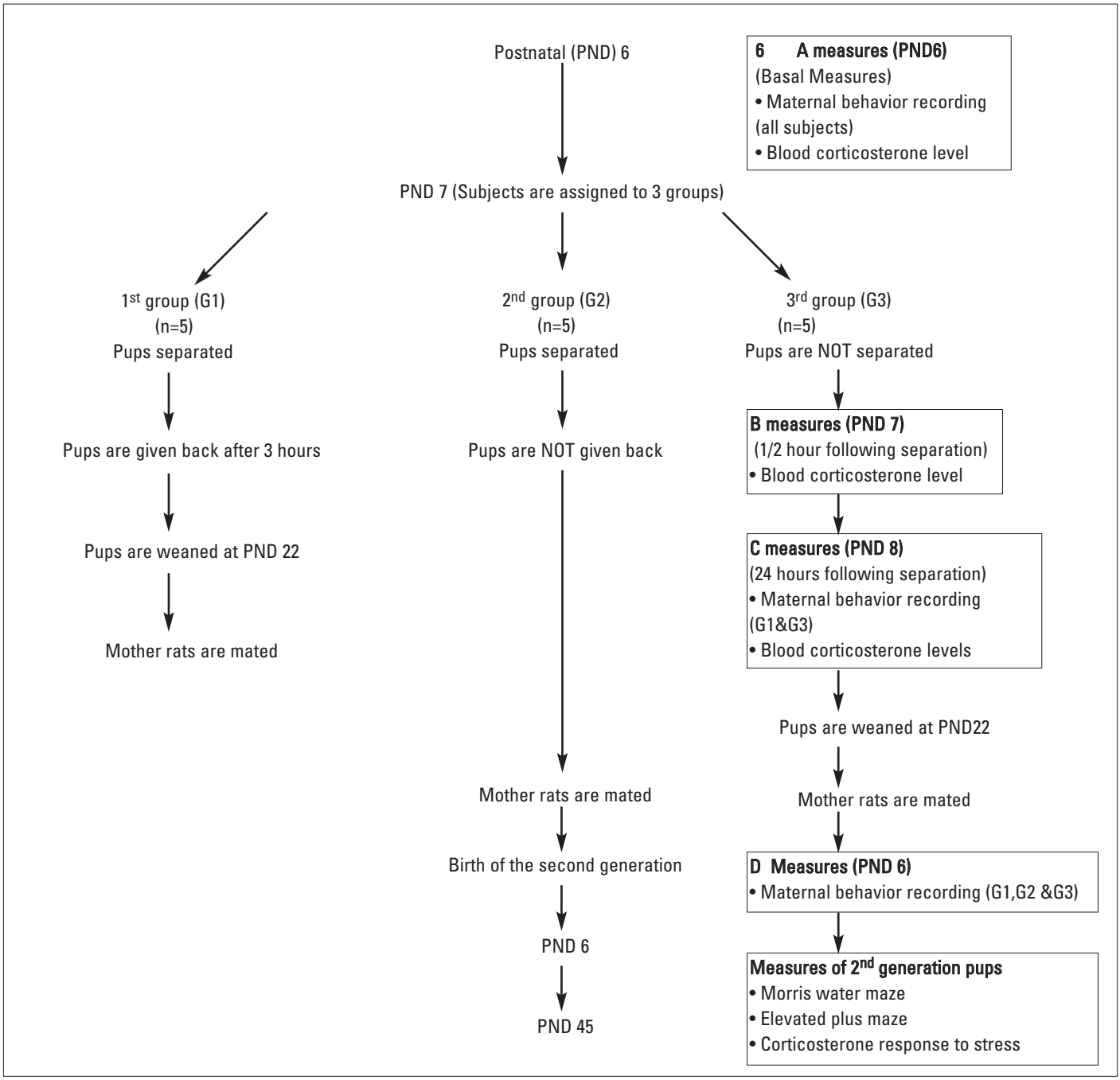

Figure 1: Design of the study indicating the schedule of the experiments. For further explanation of each experimental testing, see text.

behavior of the rat is also regulated by learned and experience-based mechanisms (22).

Additionally, blood corticosterone levels were determined in a different set of subjects (n=5 for PND 6). Results obtained at PND 6 were considered as the basal measures (A measures in Figure 1).

Maternal Behavior

Non-human mammalian mothers exhibit a synchronized set of responses to their infants to ensure that they receive adequate care to promote their social, behavioral and physiological development. The mother rat constructs a nest before giving birth. Following the birth, she carries the pups to the nest one by one, sniffs and licks the pups, especially the anogenital regions, and then ideally adopts a crouching posture over them to promote feeding (22). While all mothers exhibit a similar pattern of maternal behavior, there are variations in the style of care toward the pups. Duration and percentage of the pups carried to the nest (retrieval time and duration), total time spent at the nest, total duration of licking and nursing as well as the nursing posture constitute some of the variations in maternal care in the rat (18).

In this study, maternal behavior was evaluated by a behavioral grid modified from Patin et al., which included 30-minute video recording and subsequent assessment of direct and indirect maternal behavior of each dam (23). Total of three episodes of maternal behavior recordings were applied to each dam during the study. All of the recordings were carried out starting at 08:30 with the removal of the pups from the nest to the opposite corner of the cage, paying special attention not to change anything in the cage and not to destroy the nest. During this procedure, the pups were manipulated with cotton wool instead of bare hand or gloves to prevent cannibalism. Duration of dam's activities directed towards the pups such as nursing (in either arched-back, 'blanket' or passive posture), pup licking and retrieving latency as well as retrieval percentage were recorded as direct maternal behavior. The total time the dam spent in the nest was also noted. In addition to the evaluation of direct maternal behavior of each dam, duration of digging the sawdust, which is stated to help the thermoregulation of the nest, was observed and recorded as indirect maternal behavior. The evaluations of the video recordings were done by two researchers blind to the groupings of the dams.

Plasma Corticosterone Assay

Quantitative corticosterone measurements were performed in a different set of dams (total n=20), as the biological correlate for the possible anxiety associated with litter loss. The dams underwent the procedure of maternal behavior assessment, similar to the dams recruited for behavioral testing, to control for the possible effects of this procedure on blood corticosterone levels. The procedures involving corticosterone measurements were initiated 90 minutes after the behavioral testing was completed since it is stated that any suppressive effects on the blood corticosterone levels in rats would diminish in 90 minutes (24).

For determination of blood corticosterone levels, the animals were rapidly decapitated, the trunk blood was collected into plain tubes and the serum was separated from the blood cells by centrifuging. Since corticosterone exhibits a circadian rhythm, the time of collection was between 09:30h and 10:30h for all subjects. The collected serum was stored at -20ºC until corticosterone assay. Corticosterone concentrations were determined using the Coat-A-Count radioimmunoassay (DPC) according to the manufacturer's instructions.

On PND 7, three groups were formed. The litters of the first two groups, G1 (n=5) and G2 (n=5), were separated from their dams, whereas the litters of the third group, G3 (n=7), were left with the dams constituting the control group of the experiment.

The pups were removed from the nest using thick cotton wool to prevent cannibalism and the dam was left in its own cage. During separation, the pups of each litter were placed in a different room, where the pups of the same dam were kept together in a small compartment filled with clean bedding and warmed by a heating pad (33±0.2°C). The pups were not fed during this period.

Half an hour following the separation, blood corticosterone measurements were obtained from a different set of animals (n=5) who also underwent half an hour separation from their litters (B measures in Figure1).

The pups from G1 were returned to the maternal cage after three hours, a duration reported to represent 'chronic separation' in rat (25). When the litter was returned, the pups were distributed evenly over the cage. This group was meant to represent the chronic separation and reunion group. The pups from G2, representing the complete separation group, were not returned to the home cage and were placed in foster care of other rats not participating in the study. Male rats were allocated into the cages of G2 dams for mating.

On PND 8, maternal behavior testing was applied to the chronic separation (G1) and the control (G3) groups. Blood corticosterone levels were measured in two sets of subjects: the first set (n=5) experiencing chronic separation similar to G1 and the second one (n=5) experiencing complete separation similar to G2 (C measures in Figure 1). The dams and the pups in G1 and G3 were left undisturbed until PND 22 when the litter was weaned and male rats were placed in the cages for mating.

Procedure for the Assessment of the Consequences for the Next Generation Born to Mothers who have Previously Experienced an Early Litter Loss

Following the birth of the second generation, maternal behavior testing was applied to all the dams on PND 6 and the results were compared to those obtained in the first generation (D measures in Figure 1). The litters were kept with the dams in all groups until PND 22, when the pups were weaned and placed in different cages. On PND 45, when the second generation pups reach the adolescent age, one male and one female rat from second generation litter of each dam were randomly selected to enter the following tests.

Morris Water Maze

Morris water maze was applied to second generation pups to assess their memory and problem-solving abilities. The animals were required to locate a submerged invisible glass platform (15x15 cm) that was 1 cm below the waterline in a 1.5 m diameter pool using ambient special cues available in the testing room (26). Between 09:00h -12:00h the rats were given a total of 20 trials for 4 consecutive days. On the following 5th day, the probe trial was applied, during which the platform was removed to determine spatial bias for platform location. For all tests, search time(s) and time spent in the quadrant where the platform was located were recorded and scored using the HVS video tracking system and computer program. It is suggested that rats with better memory and problem-solving abilities would spent more time at the target quadrant during the probe trial (26).

Elevated Plus Maze

Anxiety and the TLA of the second generation pups were studied with an elevated plus maze, which was located 50 cm above the floor. The four arms of the maze is 50x10 cm and the walls of the two closed arms are 40 cm high. The experiment started with placing the animal at the cross of arms facing a close arm. Its behavior was recoded with the HVS video tracking system for 10 minutes. The time spent in the open and closed arms, the total number of entries to arms (TEA), and the TLA were scored. It is accepted that more anxious animals would spend less time in the open arms and total entries to the open arms (TEOA) would be lower (8).

Blood Corticosterone Reaction to Stress

Blood corticosterone levels of the second generation pups were measured to determine their biological reactions to stress. Since being in a novel open field is described as a psychogenic stressor for rats (27), the animals were kept in the open-field apparatus for 20 minutes prior to the procedures regarding blood corticosterone measurements.

Statistical Analysis

Since the distribution of data was not symmetrical, the Friedman analysis of variance and the Kruskall-Wallis test were applied to evaluate potential differences in maternal behavior and blood corticosterone levels. Data were assessed as repeated measures within a group as well as comparison of three different groups at a given PND. Additionally, the origins of significant difference within-group and between-group comparisons were determined conducting the Wilcoxon sign-rank test and Mann-Whitney U test, respectively.

For multi-group comparisons of data from the second generation pups, nonparametric Kruskal-Wallis test was used. Gender differences were also considered. Similarly, Mann-Whitney U test was conducted for two sets of data to determine the origin of significant differences.

The level of significance (p-value) was less than 0.05 for all tests.

Results

Dams' Reactions to Loss

Maternal behavior

Durations of dam's maternal behaviors, results of repeated measures analysis and between-group comparisons

Table 1. Duration of maternal behavior at various stages of the experiment

Group name	Stage of measurement	Duration of Pup licking (sec)	Duration of Nursing (sec)	Time spent in the nest (sec)	Retrieval Time (sec)	Duration of Digging (sec)
		Mean (min-max)	Mean (min-max)	Mean (min-max)	Mean (SD) (min-max)	Mean (SD) (min-max)
	A	160.4	309.4	557.2	274.6	52.6
		(33.0-394.0)	(0.0-788.0)	(98.0-815.0)	(60.0-803.0)	(0.0-181.0)
G1	C	264.2	659.4	955.2	76.8	11.8
		(0.0-523.0)	(50.0-850.0)	(8220.-1068.0)	(37.0-190.0)	(9.0-16.0)
	D	39.0	225.2	572.2	165.0	26.8
		(0.0-88.0)	(0.0-1014.0)	(153.0-1037.0)	(58.0-422.0)	(0.0-68.0)
G2	A	99.0	164.4	385.0	277.8	58.6
		(67.0-125.0)	(0.0-305.0)	(162.0-519.0)	(82.0-803.0)	(5.0-166.0)
	D	165.6	225.6	631.8	134.2	7.2
		(49.0-349.0)	(0.0-933.0)	(366.0-951.0)	(60.0-363.0)	(0.0-22.0)
G3	A	86.9	136.9	430.4	291.6	95.6
		(5.0-233.0)	(0.0-469.0)	(0.0-723.0)	(58.0-865.0)	(0.0-255.0)
	C	277.3	375.9	595.1	219.9	83.3
		(44.0-688.0)	(27.0-947.0)	(240.0-575.0)	(32.0-900.0)	(5.0-291.0)
	D	144.4	376.7	584.0	411.7	104.0
		(12.0-268.0)	(32.0-806.0)	(282.0-903.0)	(115.0-1177.0)	(0.0-345.0)

A: Basal maternal behavior for

C: Maternal behavior at 24 hours following separation from pups.

D: Maternal behavior at the second generation

regarding maternal behavior are summarized in Table 1, 2 and 3, respectively.

Within-group and between-group comparisons displayed that the G1 rats spent significantly more time in their nets following reunion with their pups (p=0.043 and p=0.019, respectively). Additionally, these rats demonstrated significantly increased pup licking in their second generation litters compared to their first generation litters (p=0.043).

Another significant finding suggests that the control group dams exhibited increased duration of nursing of their second generation litters compared to the first generation litters (p=0.021), whereas no such difference was observed in nursing duration for the other groups experiencing either transient or complete separation from their previous litters.

Other within-group and between-group comparisons regarding direct and indirect maternal behaviors, including retrieval percentages and being 100% for all of the rats, did not display any significant differences.

Blood Corticosterone Levels

Table 2 displays the blood corticosterone levels of the dams obtained at baseline, half an hour following separation (acute separation), 24 hours following separation and reunion (chronic separation and reunion), and 24 hours following separation without reunion (complete separation).

The results revealed that the blood corticosterone levels in the dams were significantly influenced by separation and reunion procedures (p=0.022). Analysis conducted to assess the origin of significance demonstrated that blood corticosterone levels significantly decreased when the dam was reunited with their pups following three-hour separation (p=0.014). More over, the blood corticosterone levels in dams remained significantly elevated if the pups were not returned (p=0.028).

Results Regarding the Second Generation

Morris Water Maze

Probe trial results displayed a highly significant difference between groups considering the percentage of time the pups spent at the target quadrant (p=0.000). Further analysis to assess the origin of difference showed that the second generation pups of dams experiencing either transient or complete separation from their first litters, spent significantly less time in the target quadrant compared to the second generation pups of the control group (p=0.000 for both groups). This finding indicates that pups born to mothers that have experienced an early loss of their previous litters had significantly impaired memory and problem-solving abilities compared to controls. These results are summarized in Table 3.

Additional analysis considering gender differences in probe trial results were conducted by Mann-Whitney U test. As presented in Table 4, both female and male second generation pups of dams that were separated from their previous litters spent significantly less time at the target quadrant during the probe trial, suggesting that they learned slower than the controls.

Table 2. Comparison of blood corticosterone levels of the dams obtained at baseline, following acute separation, chronic separation and reunion and complete separation

Stage of measurement	Mean (SD)	95% Confidence Interval for Mean		p*
		Lower Bound	Upper Bound	
A) Baseline	292.1 (170.6)	80.3	504.0	
B) Half an hour following separation (Acute separation)	451.0 (70.9)	338.1	563.9	
C) 24 hours following separation and reunion (chronic separation and reunion)	122.2 (95.9)	3.1	241.3	0.022#
D) 24 hours following separation without reunion (Complete separation)	306.5 (125.8)	150.4	462.7	

SD: Standard Deviation

*Kruskal Wallis Test

Mann-Whitney U test displayed that the significant difference of the means originates from the differences between stages B & C (p=0.014) and C & D (p=0.028)

Table 3. Comparison of probe trial results of second generation pups in Morris water maze

Second generation pups of groups	% time in target quadrant Mean (SD)	95% Confidence Interval for Mean		p*
		Lower Bound	Upper Bound	
G1	24.5 (6.2)	21.4	27.6	
G2	28.7 (2.1)	24.2	33.3	0.000#
G3	40.1 (2.0)	36.0	44.2	

G1: Chronic separation and reunion group,
G2: Complete separation group
G3: Control group
SD: Standard Deviation
* Kruskal Wallis Test
# Mann-Whitney U test displayed that the significant difference of the means originates from the differences between G1 & G3 (p=0.000) and G2 & G3 (p=0.000)

Elevated Plus Maze

TLA, TEA and TEOA were not significantly different for the second generation pups of G1, G2 and G3 dams (p=0.511, p=0.964, p=0.200, respectively) No significant gender differences in TLA, TEA and TEOA were observed (for males: p=0.321, p=0.389 and p=0.122, respectively and for females: p=0.521, p=0.512 and 0.617, respectively).

Corticosterone Response to Stress

There was no a statistically significant difference between the second generation pups of the three groups regarding their corticosterone responses to a psychogenic stress as summarized in Table 5. Additional analysis conducted to assess any gender differences did not yield any significant differences (p=0.471 for males, p=0.319 for females)

Discussion

The purpose of the present study was to explore how an animal model system can be used to study the reactions of postparturient female rats to the loss of their litters. Additionally, the consequences in the next litter born to mothers who have previously experienced an early litter loss are assessed to further study the nature of loss-an issue at the interface of the biological, social and medical sciences.

The results of the present study indicate that maternal behavior of rat mothers could be influenced from being separated and reunited with their pups, although their reactions are not diffuse to significantly affect each type of maternal behavior. The most prominent change was observed when the dams were reunited with the pups following three-hour separation-a duration suggested to represent chronic separation for rodents (25). Mother rats spent significantly longer time at the nest as an acute reaction to reunion. Additionally, they displayed an increase in the duration of pup licking when they took care of their second litters; a finding which could be considered as a persisting effect. On the other hand, experiencing a disruption in the mother-infant relationship seemed to had a negative impact on the duration of nursing of the pups in the second litter, during which these dams failed to exhibit the increased duration of nursing observed in the control group.

Search of literature revealed little information about how mother rats react to being separated from their pups. It has been reported that brief (approximately 15 minutes) daily separations from pups were associated with an increased maternal behavior, especially pup-licking, while extended long-term separations (3 to 6 hours) led to a decrease in the maternal behavior (18,28). In contrast to this finding, Pryce and Feldon revealed that early deprivation for 4 hours led to a marked increase in licking and arched-back nursing of the pups (29). Their findings were supported by Marmendal et al. and Zhao & Ming who also suggested that enhanced maternal behavior in response to pup-separation served as a 'compensatory' mechanism for deprivation (30,31).

These evidences, along with data from the present research, might be suggesting that preweaning pup separation could be enhancing at least some of the maternal behavior in rats and diminishing others. More over, our results indicate that effects of pup separation on maternal behavior, such as pup licking and nursing, persist for the second litter. Persistence of the changes observed in maternal behavior across several litters were also observed in a set of experiments conducted by Champagne and Meaney who have suggested that environmental stressors exerted during the gestational period and resulted in reduced maternal licking/grooming (LG) in initially high LG mothers in two subsequent litters (12). Moreover, they noted that the effects of gestational stress were observed to be selective.

Table 4. Comparison of Morris water maze probe trial results of second generation pups considering gender differences

Gender	Groups Compared	p*
Male	G1 vs. G2	0.121
	G1 vs. G3	0.001
	G2 vs. G3	0.041
Female	G1 vs. G2	0.408
	G1 vs. G3	0.003
	G2 vs. G3	0.008

G1: Chronic separation and reunion group,
G2: Complete separation group
G3: Control group
*Mann-Whitney U test

Table 5. Blood corticosterone levels of the second generation pups in response to psychogenic stress

Groups compared	Mean (SD) levels of blood corticosterone of each group	Total Mean (SD)	95% Confidence Interval for Mean		p*
			Lower Bound	Upper Bound	
G1	529.8 (105.9)				
G2	455.1 (152.3)	417.1 (178.1)	753.5	39.4	0.622
G3	549.9 (101.1)				

G1: Chronic separation and reunion group
G2: Complete separation group
G3: Control group
SD: Standard Deviation
* Kruskal-Wallis Test

There is a lack of sufficient evidence on which type of maternal behavior is selectively altered as well as on the reasons for the occurrence of such alterations in response to separation from the pups and for the persistence of these alterations across generations. Nevertheless, it can be speculated that mother rats, just like humans, could be 'recalling' the stressful life event of loosing their previous litter and might be 'afraid' of loosing the new litter, causing increased physical contact as pup licking but diminished nursing, which anxious human mothers also find very difficult to achieve (32). Maternal behavior of these 'anxious female rats' could be altered in the following litters since it is well-documented that stress alters the quality of maternal care in rodent mothers just like in human mothers (21,30,33).

Along with behavioral consequences, loss is characterized by intense emotional states induced by the disruption of the attachment bond and by perception of unmet needs in humans with accompanying stress reactions (10). Remarkably, the impact of separations on rat mothers' emotionality has been studied scarcely and biological correlates for the possible emotional reactions received almost no interest. In the only study addressing this issue, Boccia et al. suggested that 3-hour separation from pups at PND 2-12 induced a depression-like state of learned helplessness in the mother rat, displayed as an increased immobility and fewer escape attempts in a given forced swimming test (20). In our study, we assessed the stress reaction by measuring the blood corticosterone levels as the biological correlate for the possible emotional stress associated with litter loss. According to our results, blood corticosterone levels of dams remarkably increased as an acute reaction to being separated from their litters. We were also able to demonstrate that high levels of blood corticosterone decreased when the dams were reunited with their pups following a three-hour separation and persisted significantly elevated if the pups were not returned. Search of literature reveals that our results regarding blood corticosterone response of dams to pup separation could be initial to display the physiological response accompanying changes in the maternal behavior. One might speculate that dams became 'anxious' when they lost their litters.

The present study also aimed to address the consequences for the offspring of being born to a mother who had previously experienced litter loss. The second litters of dams experiencing either transient or complete separation from their first litters, displayed significant impairments in their memory and problem-solving abilities compared to the second litters of no-separation group. On the other hand, anxiety and the TLA evaluated with elevated plus maze and blood corticosterone levels in response to a psychogenic stress did not seem to be significantly altered for the second litters of the three groups.

Search of literature provided information that early-life stress in rats is reflected in cognitive change in the pups; though the data obtained from different research studies can be controversial. Some researchers report cognitive impairment such as low level of exploration, inability to process the relevant environmental cues, lower spatial learning capabilities and altered long-term memory in relation to gestational stress, while others have stated that early trauma does not cause a generalized cognitive decline at older age, but rather drives cognitive performance to the extremes, enhancing reference memory performance and spatial learning abilities (8,11,33-36). The reasons for these discrepancies are not readily apparent but may be due to the intensity, duration and timing of the maternal stress, the age of the rats when tested, and how learning and memory were measured.

Regarding our results of cognitive testing, it is intriguing that the second generation pups of both separation groups have displayed significant deficits in their memory and problem-solving abilities. It could be suggested that separation from the previous litter, by causing stress in the mother rat and altering maternal behavior as reflected by diminished nursing, might have affected the cognitive abilities of second generation pups. The results of the present study show alterations in blood corticosterone levels of dams in response to separation from their first litter. Unfortunately, the design of the study falls short to provide data regarding the blood corticosterone levels of dams during the gestational period, after which the second litters were born. Considering their anxiety levels and lack of elevation of blood corticosterone in response to psychogenic stress, it is difficult to suggest that the cognitive deficits observed in the second generation pups may only be related to blood corticosterone alterations in the dams.

The question of 'how previous litter loss could have effected the cognitive abilities of the next litter?' might be better inquired by the inclusion of 'bio-communicators', as termed by Tozuka et al., in the study design (37). Nuclear factor kappa B, which is suggested to play a regulatory role in prenatal stress-induced cognitive impairment, maternal vasoactive intestinal peptide, ghrelin, and oxytocin, known to be related to emotional stress in the mother rat and affecting the neural development of offspring as well as Protein Kinase C beta1 in the hippocampus resulting from prenatal restraint stress suggested to play an important role in the impairment of learning and memory abilities of offspring could be examples for further research (34,38,39). Additionally, measurement of hippocampal long-term potentiation is considered to be a reliable indicator of the direction of neurophysiologic changes underlying learning and memory induced by maternal stress of different intensities (35).

Although the presented results of the study are intriguing, the limitations of the study require caution when generalizing the results. The experiments should be replicated with a larger sample size since the present study could be underpowered due to the limited sample size. We observed significant changes in maternal behavior in the second litters of dams experiencing separation from their first litters, nevertheless, the present research failed to provide clear evidence that rat mothers experienced gestational stress due to separation. Additionally, our intention to compare maternal behavior at the same postnatal time in both generations and PND 6 being chosen in compliance with research stating that maternal behavior is regulated by learned and experience-based mechanisms rather than hormonal pathways solely following this period, might have led to a design flaw to assess the influences of maternal care on the cognitive abilities of the second litters since some research studies indicate that maternal care in the first postnatal week can be critical for the offspring development (22).

In summary, the present research provides initial preliminary evidence that rat mothers also display physiological and behavioral responses to being separated from their litters. Additionally, disruption of early and intimate relationships seems to have adverse effects on the cognitive capacities of the following litter, pointing to the existence and importance of a complex system of mutual physiologic, behavioral and emotional interaction between mother and infant with transgenerational effects. Analysis of the process of loss in other animal models, known to be particularly useful in narrowing the gap between psychology and the physiological processes underlying behavior and emotions, may stimulate further clinical research with probable clinical implications for bereaved mothers, which as a result would be of great benefit both for the mothers and the infants.

Acknowledgments

We are grateful to Hakan Baydur, PhD for his contributions to the statistical analysis of our data.

This research was supported by a grant from The Scientific and Technical Research Council of Turkey awarded to A. Ozbek.

References

1. Forstmeier S, Maercke A. Comparison of two diagnostic systems for Complicated Grief. J Affect Disorder 2007; 99:203-11. [Abstract] / [Full Text] / [PDF]
2. Hughes PM, Torton P, Evans GDH. Stillbirth as a risk factor for anxiety and depression in the next pregnancy: Does time since loss make a difference: A cohort study. BMJ 1999; 318:1721-24.
3. Kumar R, Robinson KM. A prospective study of emotional disorders in childbearing women. Br J Psychiatry 1984; 144:35-47. [Abstract] / [PDF]
4. Badenhorst W, Hughes P. Psychological aspects of perinatal loss. Best Pract Res Clin Obstet Gynaecol 2006; 21:249-59. [Abstract] / [Full Text] / [PDF]
5. Teixeira JM, Fisk NM, Glover V. Association between maternal anxiety in pregnancy and increased intrauterine artery resistance index: cohort based study. BMJ 1999; 318:153-7. [Abstract] / [Full Text] / [PDF]
6. O'Connor TG, Heron J, Golding J et al. Maternal antenatal anxiety and children's behavioural/emotional problems at 4 years. Report from the Avon Longitudinal Study of Parents and Children. Br J Psychiatry 2002; 180:502-8. [Abstract] / [PDF]
7. Glover V, O'Connor TG. Effects of antenatal anxiety. Br J Psychiatry 2002; 180:389-91. [Full Text] / [PDF]
8. Lordi B, Patin V, Proatis P et al. Chronic stress in pregnant rats: effects on growth rate, anxiety and memory capabilities of the offspring. Int J Psychophysiol 2000; 37:195-205. [Abstract] / [Full Text] / [PDF]
9. Mulder EJ, Robles de Medina PG, Huizink AC et al. Prenatal stress: The effects on pregnancy and the (unborn) child. Early Hum Dev 2002; 70:3-14. [Abstract] / [Full Text] / [PDF]
10. Hofer MA. On the Nature and consequences of early loss. Psychosom Med 1996; 58:570-81. [Abstract] / [PDF]
11. Kloet RE, Oiltz MS. Who cares for a stressed brain? The mother, the kid or both. Neurobiol Aging 2003; 24:61-5. [Abstract] / [Full Text] / [PDF]
12. Champagne FA, Meaney MJ. Transgenerational effects of social environment on variations in maternal care and behavioral response to novelty. Behav Neurosci 2007; 121:1353-63. [Abstract]
13. Levine S. Primary social relationships influence the development of the hypothalamic-pituitary-adrenal axis in the rat. Physiol Behav 2001; 73:255-60. [Abstract] / [Full Text] / [PDF]
14. Champagne FA, Francis DD, Mar A et al. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiol Behav 2003; 79:359-71. [Abstract] / [Full Text] / [PDF]
15. Cannizzaro E, Martire M, Gagliano M et al. Reversal of prenatal diazepam-induced deficit in a spatial-object learning task by brief, periodic maternal separation in adult rats. Behav Brain Res 2005; 161:320-30. [Abstract] / [Full Text] / [PDF]
16. Kuhn C, Schanberg SM. Responses to maternal separation: mechanisms and mediators. Int J Dev Neuroscience 1998; 16:261-70. [Abstract] / [Full Text] / [PDF]
17. Kalinichev M, Easterling KW, Holtzman SG. Early neonatal experience of long-evans rats results in long-lasting changes in reactivity to a novel environment and morphine-induced sensitization and tolerance. Neuropsychopharmacology 2002; 27:518-33. [Abstract] / [Full Text] / [PDF]
18. Lovic V, Gonzalez A, Fleming AS. Maternally separated rats show deficits in maternal care in adulthood. Dev Psychobiol 2001; 39:19-33. [Abstract] / [PDF]
19. Bredy TW, Humpartzoomian RA, Cain DP et al. Partial refusal of the effects of maternal care on cognitive function through environmental enrichment. Neuroscience 2003; 118:571-6. [Abstract] / [Full Text] / [PDF]
20. Boccia ML, Razzoli M, Vadlamudi SR et al. Repeated long separations from pups produces depression-like behavior in rat mothers. Psychoneuroendocrinology 2007; 32:65-71. [Abstract] / [Full Text] / [PDF]
21. Lewis E. Stillbirth Psychological consequences and strategies of management. Editörler Mkunsky A, Friedman EA, Gluck L. In: Advances in perinatal medicine. Plenium Publishing Co; 1988; s. 206-45.
22. Fleming AS, O'day DH, Kraemer GW. Neurobiology of mother-infant interactions: experience and central nervous system plasticity across development and generations. Neurosci Biobehav Rev 1999; 23:673-85. [Abstract] / [Full Text] / [PDF]
23. Patin V, Lordi B, Vincent A et al. Effects of prenatal stress on maternal behavior in the rat. Dev Brain Res 2002; 139:1-8. [Abstract] / [Full Text] / [PDF]
24. Rimondini R, Agren G, Börjesson S et al. Persistent behavioral and autonomic supersensitivity to stress following prenatal stress exposure in rats. Behav Brain Res 2003; 140:75-80. [Abstract] / [Full Text] / [PDF]
25. Mc Intosh J, Anisman H, Merali Z. Short and long periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: Gender-dependent effects. Dev Brain Res 1999; 113:97-106. [Abstract]
26. Morris RG, Garrud P, Pawlins JN et al. Place navigation impaired in rats with hypocampal lesions. Nature 1982; 297:681-83. [Abstract]
27. Anisman H, Hayley S, Kelly O et al. Psychogenic, neurogenic and systemic stressor effects on plasma corticosterone and behavior: mouse strain-dependent outcomes. Behav Neurosci 2001; 115:443-54. [Abstract] / [PDF]
28. Boccia ML, Pedersen CA. Brief vs. long maternal separation in infancy: Contrasting relationships with adult maternal behavior, lactational levels of aggression and anxiety. Psychoneuroendocrinology, 2001; 26:657-72. [Abstract] / [Full Text] / [PDF]
29. Pryce CR, Feldon J. Long-term neurobehavioural impact of the postnatal environment in rats: manipulations, effects and mediating mechanisms. Neurosci Biobehav Rev 2003; 27:57-71. [Abstract] / [Full Text] / [PDF]
30. Marmendal M, Roman E, Peter Eriksson PCJ et al. Maternal separation alters maternal care, but has minor effects on behavior and brain opioid peptides in adult offspring. Dev Psychobiol 2004; 45:140-52. [Abstract] / [PDF]
31. Zhao C, Ming L. Sedation and disruption of maternal motivation underlie the disruptive effects of antipsychotic treatment on rat maternal behavior. Pharmacol Biochem Behav 2009; 92:147-56. [Abstract] / [Full Text] / [PDF]
32. Zelkowitz P, Papageorgiou A. Maternal anxiety: an emerging prognostic factor in neonatology. Acta Paediatr 2005; 94:1704-5. [Abstract] / [Full Text] / [PDF]
33. Hosseini-Sharifabad M, Hadinedoushan H. Prenatal stress induces learning deficits and is associated with a decrease in granules and CA3 cell dendritic tree size in rat hippocampus. Anat Sci Int 2007; 82:211-7. [Abstract]
34. Li H, Li X, Jia N et al. NF-kappaB regulates prenatal stress-induced cognitive impairment in offspring rats. Behav Neurosci 2008; 122:331-9. [Abstract] / [PDF]
35. Yaka R, Salomon S, Matzner H et al. Effect of varied gestational stress on acquisition of spatial memory, hippocampal LTP and synaptic proteins in juvenile male rats. Behav Brain Res 2007; 179:126-32. [Abstract] / [Full Text] / [PDF]
36. Barha CK, Pawluski JL, Galea LA. Maternal care affects male and female offspring working memory and stress reactivity. Physiol Behav 2007; 92:939-50. [Abstract] / [Full Text] / [PDF]
37. Tozuka Y, Wada E, Wada K. 'Bio-communication' between mother and offspring: lessons from animals and new perspectives for brain science. J Pharmacol Sci 2009; 110:127-32. [Full Text] / [PDF]
38. Handa RJ, Zoeller RT, McGivern RF. Changes in vasoactive intestinal peptide and arginine vasopressin expression in the suprachiasmatic nucleus of the rat brain following footshock stress. Neurosci Lett 2007; 425:99-104. [Abstract] / [Full Text] / [PDF]
39. Wu J, Song TB, Li YJ et al. Prenatal restraint stress impairs learning and memory and hippocampal PKCbeta1 expression and translocation in offspring rats. Brain Res 2007; 141:205-13. [Abstract] / [Full Text] / [PDF]