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Behavioral and Neurodevelopmental Effects of Early Interventions in Adult Wistar Rats

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ABSTRACT

Introduction: Interventions performed in the early period of life are associated with cognitive and behavioral changes in adulthood. The effects of interventions such as exposure to an early stressful life event or environmental enrichment on cognitive and behavioral development are studied. The aim of this study is to develop a new intervention method, to investigate the effects of early interventions on social interaction, memory anxiety levels and NR2B levels in prefrontal and hippocampus in adulthood. The hypothesis of the study is that exposure to the ambivalent mother will affect the behavioral performance of rats at least as much as one hour apart from the mother in the adult period and cause changes in the prefrontal cortex and hippocampus in the NR2B levels.

Method: In the study, the Wistar rats were divided into four groups as control group (12), group that remained 15 minutes apart from mother (Mild Stress) (12), group that remained 60 minutes apart from mother (Severe Stress) (14) and ambivalent mother group (13). In adulthood, the social interaction test, elevated plus maze and new object recognition test performances of rats were evaluated. ELISA method was used to evaluate the effect of interventions on the prefrontal cortex and hippocampus NMDA R2B levels.

Results: The important findings of the study were that in the new object recognition test, separation from the mother did not affect the recognition memory regardless of gender, while the short-term recognition memory of the females of the ambivalent mothers' group was better than the females in the other groups and the long-term memory performance of the mild stress groups men was better than the man in the other groups. In addition, in the social interaction test, the males of the ambivalent group and the mild stress group showed more aggressive behavior. It was determined that the prefrontal cortex NR2B level was higher in the mild stress and ambivalent mother group compared to the severe stress group, and NR2B level was increased in all intervention groups compared to the control group.

Conclusion: In contrast to the hypothesis, the results of this study support that the ambivalent mother's group rats are not more adversely affected than the severe group rats and that the model created may be an environmental enrichment model rather than an early stressful life event exposure.

Keywords: Wistar rats, behavioral manipulations, NR2B NMDA receptor, aggression

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INTRODUCTION

Early life is the most important era, which is open to external factors in terms of the development of the individual. In this period, exposure to stressful life events is generally associated with increased stress sensitivity, cognitive and behavioral disorders in adulthood (1, 2). It is possible through animal studies rather than human studies to examine and control the long-term behavioral and biochemical effects of a particular stressor exposed.

Hofer argues that a series of sensorimotor, thermal and feeding-based events, which are components of typical parental and infant interactions, have long-term regulatory effects on infant behavior and physiology (3). Among these regulatory factors, deficiencies that arise due to parental deprivation or parental loss cause deficiencies such as circadian rhythm, growth (including brain growth factors) (4) and hormone levels (including HPA activity) (5). In the studies on rats, the duration of mother deprivation is classified as short-term for 10 to 60 minutes and as long-term maternal separation for the periods ranging from 60 to 480 minutes (6). The time factor is significant because some studies have reported that short separation times do not impair the development of offspring, but the prolonged separation may result in an inadequate environment for neuro-development (7.8). Furthermore, it is known that the synaptogenesis in which the rat brain is not fully developed at birth is at the maximum level in postnatal 4-11th days and hippocampal neuronal cell development and dendritic development in postnatal 5-7th days. Therefore, early mother separation interventions are implemented in the first two weeks of life.

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It is a known fact that the key molecule in the process of neurodevelopment is N Methyl D Aspartate (NMDA), which is one of the ionotropic glutamate receptors necessary for synaptic plasticity and excitatory neurotransmission. NMDA receptors are structures that are formed by the combination of NR1 and NR2 sub-units at divergent rates. One of these sub-units is NR2B. The regions where NR2B expression is most intense are cortex> olfactory bulbus> hippocampus (9). NR2 can increase memory and cognition in many forms of memory, including sensory memory, associative memory, recognition memory, and social memory (17). The NR2B sub-unit has been shown to be expressed extensively concerning the social recognition mechanism in the frontal brain regions, including the olfactory bulb, amygdala, cortex and hippocampus, which are involved in olfactory learning and olfactory memory (10). It has been depicted that the adult NR2B mRNA level of rats, which remained separated from the mother for a daily period of 15 minutes in the early life, increased in the prefrontal cortex and dorsal hippocampus (11). In a study, it was reported that there was a decrease in NMDA NR2B mRNA expression in the hippocampus in male adult rats exposed to MS and that no change occurred in the prefrontal cortex (12).

Studies have shown that separation from the mother early in life leads to changes in the behavior of adult rats. Adult rats who have been exposed to a short-term separation intervention from the mother have been reported to feel less fearful, less emotional and more discovery-oriented when entering a new environment (13). In elevated t-maze test implemented to evaluate anxiety behavior, it is shown that rats spend more time in the open arms of the maze, that they can distinguish new and familiar objects in the social learning test, but that they make fewer social discoveries during the learning phase, and further, during the social interaction test, it was observed that participatory social behaviors such as sniffing decreased and non-participatory behaviors such as aggression increased (14). Although there are studies concluding that adults rats separated from their mothers for a long time during the first 3 weeks after birth did not show any difference in behavior from the control group, there are studies reporting that they spend less time in the open arms of the elevated T-maze and fail in the new object recognition test compared to the control group (15,16,17).

When the literature is analyzed, it is observed that whereas the absence or deprivation of the mother is considered as exposure to stress in early interventions, the information regarding the effect of the ambivalent conditions such that the mother does not feed or does not give care to the offspring in that period is insufficient. In humans, it is thought that the mother's sometimes loving, sometimes cold and indifferent behavior towards the child may be a risk factor for the emergence of many psychiatric disorders when the child reaches adulthood. It is difficult to evaluate the behavioral effects of exposure to ambivalent behavior of the caregiver in early adulthood alone in human studies. Studies can generally be planned considering that the current parental attitude was ambivalent also in the past. Accordingly, the results obtained may also not reliable due to many confusing factors.

The primary purpose of this study is to develop a new early intervention model with an ambivalent mother who does not care and feeds the offspring. The second aim is to examine the effects of early stressful life events on social interaction, memory, anxiety level and neuro-development of the hippocampus and prefrontal cortex in adulthood. The hypothesis of the study is that exposure to an ambivalent mother will adversely affect the behavioral performance of rats in adulthood, at least as in rats separated from the mother for one hour, and that this intervention will also cause changes in the levels of NR2B in the prefrontal cortex and hippocampus, which is thought to be associated with recognition memory, social memory and olfactory memory.

METHOD

Provision and study of rats

This study gained Ethics Committee approval with the decision numbered GU-ET-14069 of Gazi University Animal Experiments Local Ethics Committee. Rats in the study were obtained from Gazi University Laboratory Animal Breeding and Experimental Research Center. In accordance with the Gazi University Ethics committee rules, 2 pregnant rats per group and 8 pregnant Wistar rats in total were obtained. The study was conducted with 51 rats (28 males and 23 females) that were born by these rats. The day of birth was accepted as day 0. Working with rat pups started from day 1 after birth. There are 6 males and 6 females in the Control Group, 6 males and 6 females in the mild stress Group, 9 females and 5 males in the severe stress group, and 5 males and 8 females in the Ambivalent Mother Group.

Interventions to Rat pups on Postnatal Days 1-14

Rats in the control group postnatal (PN) grew on the 1-14th days without any intervention with their mothers (non-handling: NH). During this period, only once every three days were entered into the environment where they were placed for the purpose of meeting their water and food needs.

The second group of rats was taken out of the cage (first the mother and then the others) every day at 10:00 am and was placed in a separate cage during PN 1-14th days. Later, with the hands without gloves they were taken out of the cage one by one and put into plastic containers filled with sawdust, with a heater at 28-30°C underneath. After waiting for 15 minutes in this container, they were put back into the cage. After this procedure was completed for all offsprings, the mother was finally put back in the cage. For the purpose of the study, this group was called "mild stress group".

In the third group, the Rat Separation Model (maternal seperation: MS) was applied to the rats in the PN 1-14th days. In this arrangement, the rat pups were removed from the cage every day immediately after the mother fed the offsprings in the morning and then were placed in a separate cage during PN 1-14th days. The mother was kept in a separate cage from the offspring for 60 minutes. During this time, the heater in the room was operated, keeping the room temperature between 25-30°C, and after 60 minutes, the rat pups were put back in the mother's cage. This group was called "severe stress group" in the study.

During PN 1-14th days, the rat pups in the fourth group were exposed to the ambivalent mother who was created by developing the ambivalent mother model that was previously used in a study (18). In this arrangement, the actual mother was removed from the cage every day at 10:00 pm (when the rats were at night cycle), and it was replaced with the "ambivalent mother" (AM). AM is an inanimate, plush object that remained with the rats from the 1st week before the birth of pregnant rats, and on which the mother's smell, urine is thought to be permeated, and which cannot function as the actual mother such as touching, licking and breastfeeding. At the end of 60 minutes, the baby rats were separated from the "ambivalent mother" and their actual mothers were put back in the cage. Figure 1 depicts the plush animal, mother and rat pups used.

Considering the development of the offsprings, they were separated from their mothers, and the male and female genders were separated on the 30th postnatal day. 3-4 rats of the same sex were placed in each cage. Each rat was given the same care, 1-2 times a week, no changes were made, except for the swap changes, once every 3 days. On the 60th day, the rats were taken to the center, where behavioral experiments would be conducted. Behavior experiments were carried out between days 67-79 after the habituation and observation process.

Figure 1. Plush rats used in the Ambivalent mother group

Behavior Test Battery

Experiments were carried out under dim light between 10:00-13:00 hours. The animals were brought to the experimental environment half an hour before the experiment started in order to have the animals got used to the environment. Detailed work schedule is depicted in Table 1.

Social Interaction Test

This test is a model developed by File and Hyde in 1978 to evaluate the effect of anxiolytic drugs. At the same time, reduced social interaction was reported by Kay SR (1987) and Sams-Dodd (1997) as the potential animal models of negative symptoms in schizophrenia. The social interaction test has been adapted according to the particular method of the study in various studies. In previous studies, it was observed that the anxiety level and plasma corticosterone levels of the rats increased under high light and unfamiliar environments. Therefore, two days before the test started, rats were left in an open box of 100x100x50 cm for 4 minutes/a day in order to get used to the environment. When the test started, two rats, which are alien to each other, with a weight difference of not more than 15 g, were placed in the experimental environment. Their behavior was recorded for 10 minutes. In order to evaluate social interaction data, the data are grouped as active social behavior (rapprochement, tracking, sniffing and social grooming, escaping, avoidance, climbing up and down), activity (traveling and examining) and stress-related behavior (self scrabbling and freezing). At the end of each test, the area was cleaned with 10% alcohol. The social interaction test was applied when the rats were 67 days old.

Elevated t-Maze Test

The configuration and testing procedure of this test was developed by Pellow and File (1986). This device is a t-shaped platform made of plexiglass, at 50 cm high, consisting of two open (50 X 10 cm) and two closed (with 50 X 10 cm and 38 cm high walls) arms and adjusted to stand against each other. The rats were placed in the middle of the device with their faces facing one of the closed arms and then the behavior of the animals was monitored for 5 minutes. Normally, the experimental animal feels safer in closed arms and spends most of its time in closed arm or arms. Medications with anxiolytic effects have been shown to increase the number of times of entrance to or to stay in the open arms. In this experiment, the number of animals entering the arms, the time they spent in the arms and the distance they traveled in the arms were monitored and recorded with the webcam-connected Video Tracking System connected and the following parameters were calculated:

Number of entrance to open arms / Percentage of entering open arm=x100 Total number of entrance to arms Time spent in open arms

Percentage of Time Spent in open arms=x100 Time spent in arms

In this study, the elevated t-Maze test was performed at the time when rats were 72 days old.

New Object Recognition Test

There are many models developed for visual recognition memory in rats. In this study, the test protocol which was defined for the first time in 1988 by Ennaceur and Delacour was applied. For this test, an opaque white open field box of 100 X 100 X 50 cm, the base of which is divided into squares smaller than itself, is used. Objects with 3 copies each of different colors and shapes made of wood were used (A1, A2, A3, B1, B2, B3, C1, C2, C3). In order for them not to remove the rats, these objects are fixed to the ground in a way that that they can be attached to the ground and then removed. A new object recognition test was applied on the 76-79th days. First of all, in order to prevent the anxiety that may occur due to entering a new environment, a practice test in the form of 2 sessions lasting 10 minutes for each animal is performed. The actual test was started 24 hours after the last practice session. All tests were recorded on the video camera. First, A1 and A2 (two identical objects) objects were placed in the box, close to each other (approximately 10 cm), to coincide with the two adjacent sides (recognition session). Then the rat was placed in the box and kept in the box for 10 minutes. After the rat was removed from the box, both of the objects were removed, and the same object (A3) and a new object (B) were placed in their place (on the same place as the previous objects). For short-term memory, the rat was put back in the box and kept in the box for 5 minutes 1 hour after the recognition session. Meanwhile, the time spent by the rat for examination in both tests was recorded. The object examination behavior is defined as bringing the object close to the nose up to 2 cm and touching it. For long-term memory, 24 hours after the recognition session, the same object (A4) and another new object (C) were placed (in the same place as the previous objects), the rat was kept in the box for 5 minutes, and the examination behavior on the objects was recorded. A rat with fine recognition memory is expected to spend more time with new objects (B and C) than older objects (A3 and A4).

In the New Object Recognition Memory Test, the time spent by the rats with the familiar object (TF), the time spent with the new object (TN) and the total time spent with both objects were recorded in seconds. Two indices were calculated using these data. Recognition Index (RI) is considered as the basic indicator of recall and is calculated as the ratio of the time of examining the new object to the total object inspection time:

TN RI = TN + TF

Another calculated index is the Discrimination Index (DI). It indicates the ability to distinguish new and familiar objects. This is a value between +1 and -1. A positive score indicates that more time is spent with the new object, and a negative score indicates that more time is spent with the familiar object, and a value of zero time means that time spent with both objects is equals. This index is calculated as follows:

TN - TF DI=TN + TF

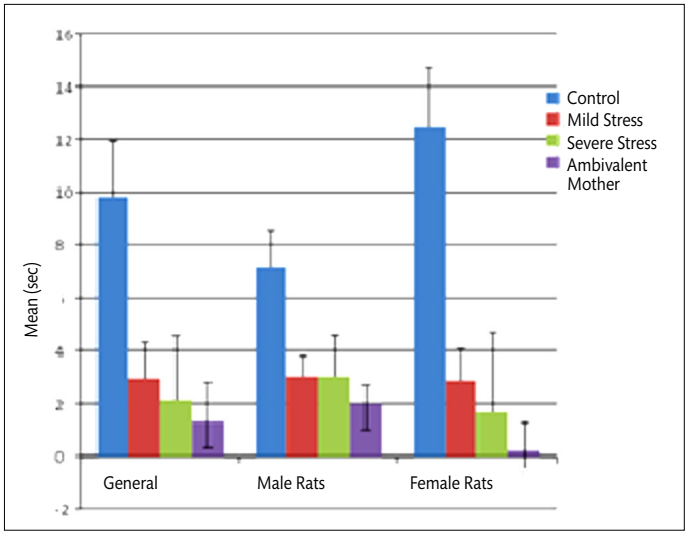
Killing of animals, collecting body blood and removing brain tissues

The brain tissues of the rats were removed following a cardiac arrest by giving the animals 30 mg/kg at the Gazi University Experimental Animal Breeding and Experimental Research Center 2 hours after the long memory evaluation session of the new object recognition memory. The prefrontal cortex and hippocampus were separated from the brain in a manner suitable for the rat brain atlas. Each tissue sample was coded into

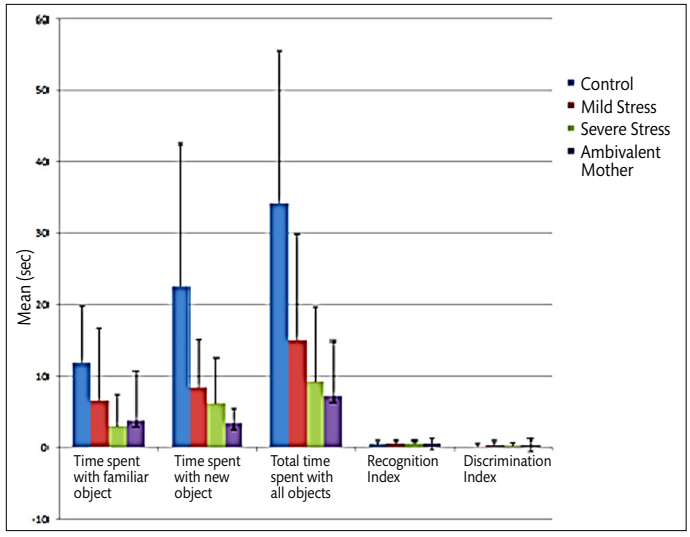
epandorphs and placed at -80 °C, passing through liquid nitrogen until the performing of ELIZA analysis. In order to measure plasma cortisol level, rat body blood was collected via an injector, and each blood was coded and taken into biochemistry tubes, and cortisol levels were examined on the same day.

ELISA Test

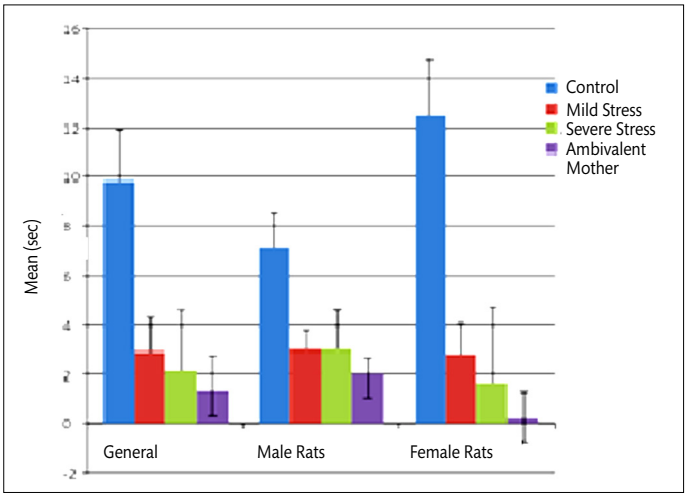
After the tissues were homogenized in homogenization buffer, the homogenates obtained were centrifuged at 14000 g for 30 minutes. The NMDAR2B formed was analyzed. NMDAR2B levels were measured using a sandwich EIA (enzyme immunoassay) method using a ready kit



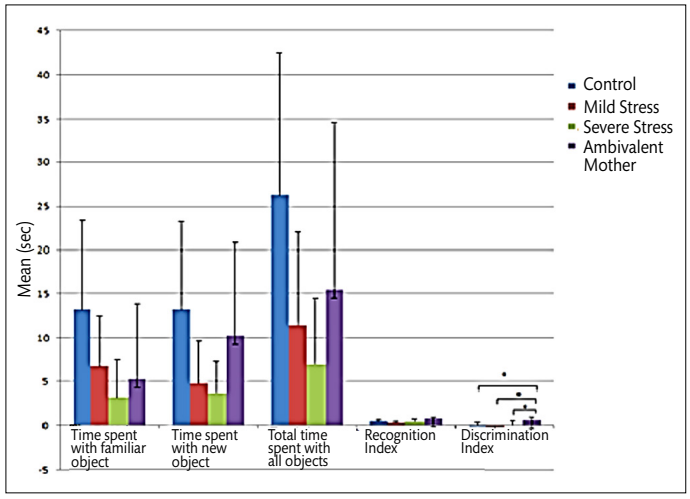
Graph 1. Duration of entering open arms results of groups



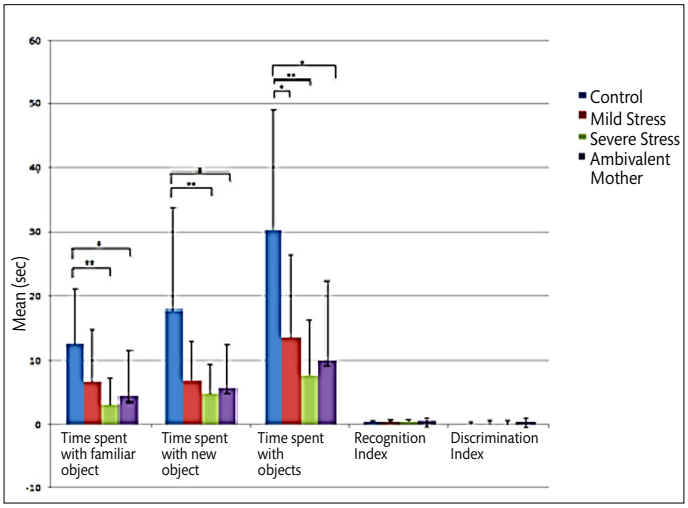
Graph 4. New object recognition memory of male animals-Short term memory results



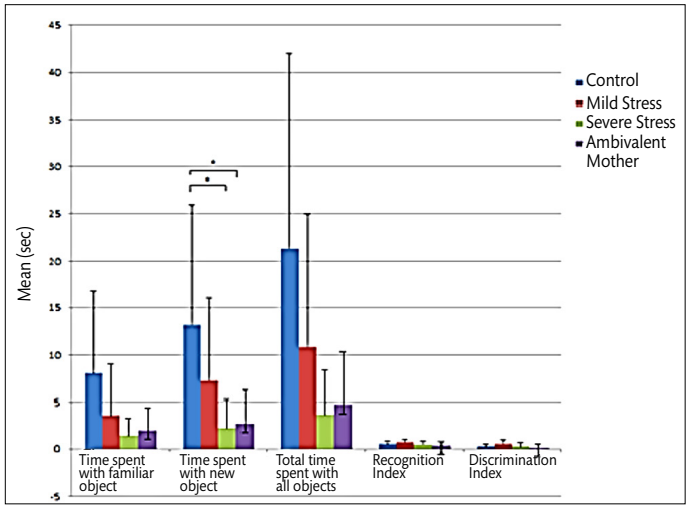
Graph 2. Duration of spending time in open arms of groups



Graph 5. New object recognition memory of female animals-Short term memory results (*p<0.05)



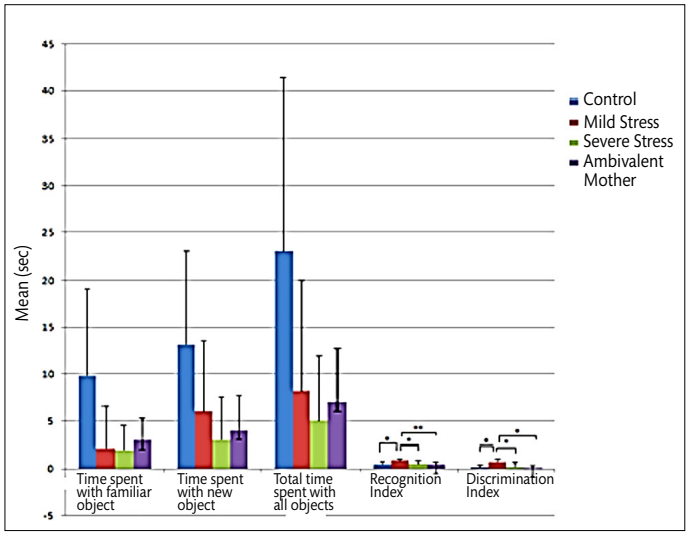
Graph 3. New object recognition memory of all groups-Short term memory results (*p<0.05, **p<0.01)



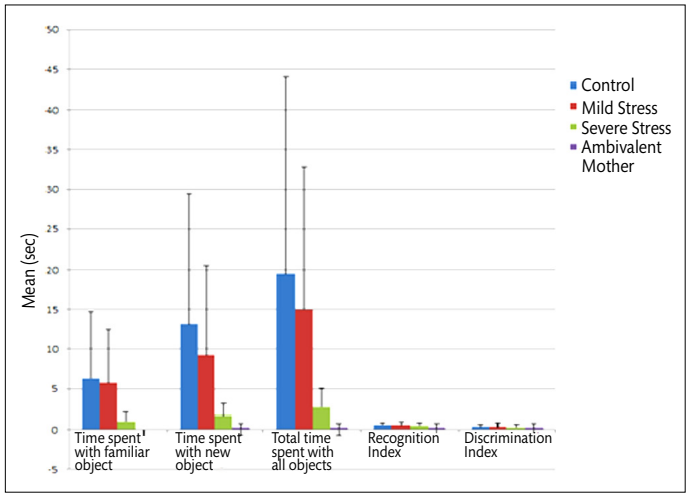
Graph 6. New object recognition memory of all animals-Long-term memory results (*p<0.05)

(NMDAR2B, Eastbiopharm). NMDAR2B analysis range was 0.5-10 ng / ml, sensitivity was 0.02 ng / ml.

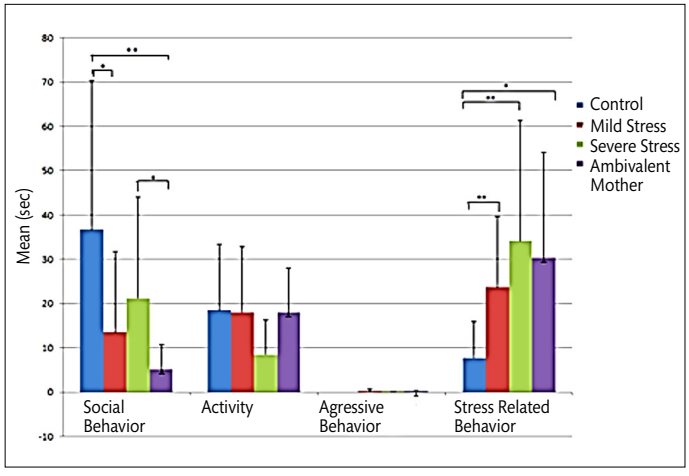
Gender distribution and rat numbers during the experiments according to the groups are depicted in Table 1.



Graph 7. New object recognition memory of male animals-Long-term memory results (*p <0.05, **p<0.01)



Graph 8. New object recognition memory of female animals-Long-term memory results



Graph 9. Social behavior performance results of all animals (*p <0.05, **p<0.01)

Statistical Analysis

Statistical analyses were performed using the Statistical Packages for the Social Sciences (SSPS) 21.0 package program. In all analyses, non-parametric tests were utilized. Kruskal-Wallis analysis in comparing between groups, and Mann Whitney U analysis in understanding which group makes the difference are used.

RESULTS

Gender comparisons of the animals included in the study are shown in Table 2.

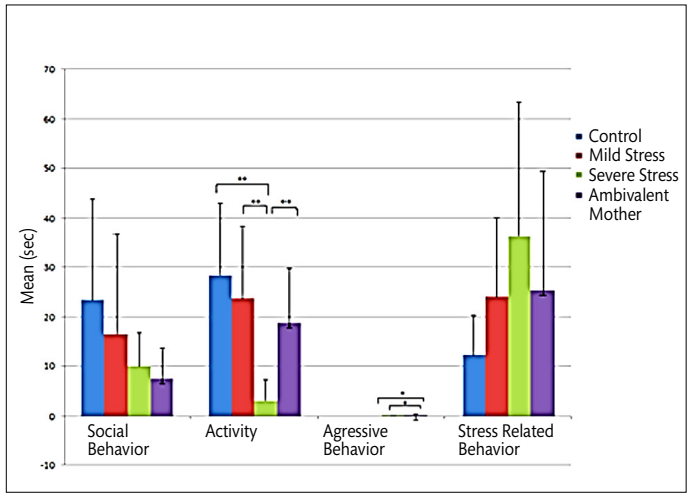
Elevated t-Maze

The groups were compared in terms of their performance in the Elevated t-Maze both in general and particularly by gender, and there was no significant difference between the groups in terms of their performances in the Elevated t-Maze.

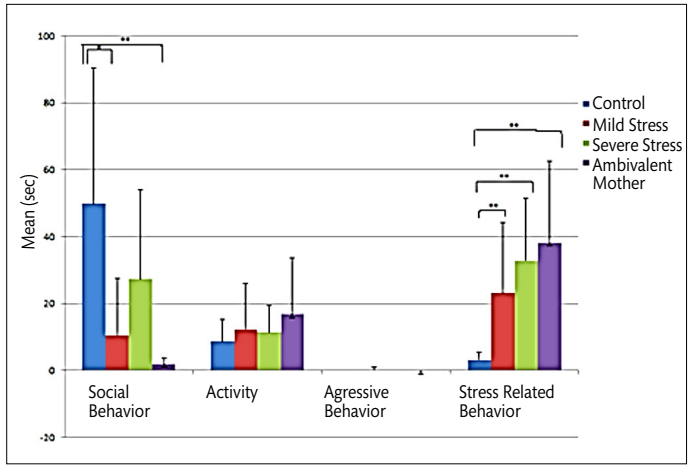
New Object Recognition Memory Test

Short Term Memory

In the evaluation without making any gender discrimination, it is found that there was a significant difference between the groups in the test for short-term memory regarding the familiar object of the groups (X^2=10.601, p<0.05), new object (X^2=9.044, p<0.05) and the total time they spent with both objects (X^2=10.619, p<0.05). There was no difference found between the groups in terms of Discrimination Index (X^2=4.285, p=0.232) and Recognition Index (X^2=3.969, p=265). As a result of the analysis made to



Graph 10. Social behavior performance results of male animals (*p <0.05, **p<0.01)



Graph 11. Social behavior performance results of female animals (*p <0.05, **p<0.01)

Table 1. Work Schedule

	Control Group	Mild Stress Group	Severe stress Group	Ambivalent Mother Group
D1-14	Standard Care	Mild Stress Group	Severe Stress Group	Ambivalent Mother Method
D14-30	Standard Care			
D31	Separation from mothers, separation of male / female rats from each other			
Gender distribution	E: 6 D: 6	E: 6 D: 6	E: 9 D: 5	E: 5 D: 8
D32-60	Standard Care			
D61	Bringing the animals to the Neuroscience Laboratory			
D62-D64	Standard Care			
D65-D66	Daily 5-minute exercise for Social Interaction Test			
D67	Social Interaction Test			
D68-71	Standard Care			
D72	Elevated t-Maze			
D72-75	Standard Care			
D76-D77	Practice in a 10-minute environment for the New Object Recognition Test			
D78	New Object Recognition Test - Short Term Memory			
D79	New Object Recognition Test- Long-term Memory, Removal of Brain Tissues			

understand the source of the difference in terms of the time spent with the familiar object, the animals in the control group (12.5±8.72), spent time with more familiar objects than animals in severe stress (3.15±4.16, Z=-2.872, p<0.01) and ambivalent mother (4.33±7.09, Z=-2.505, p<0.05) group. As a result of the analysis made to understand the source of the difference in terms of the time they spent with the new object, the animals in the control group (17.83±15.92) spent significantly more time with new objects than animals in the severe stress (4.69±4.68, Z=-2.788, p<0.01) and ambivalent mother (5.67±6.65, Z=-2.401, p<0.05) group. Finally, in terms of the total time spent with both objects, animals in the control group (30.33±18.54) were found to spend more time with both objects than animals in mild stress (13.6±12.75, Z=-2.080, p<0.05), severe stress (7.85±8.38, Z=-2.942, p<0.01) and AM (10±12.36, Z=-2.340, p<0.05) groups.

In the evaluation made for male animals, no significant difference is found in terms of time spent with intra-group familiar object (X^2=3.838, p=0.279) and new object (X^2=6.233, p=0.101) and the total time they spent with both objects (X^2=5.286, p=0.152), Discrimination Index (X^2=1.241, p=0.43) and Recognition Index (X^2=1.277, p=0.735).

As a result of the analysis performed for female animals, while there ise a significant difference in terms of the Discrimination Index (X^{2nd}=8.307, p<0.05) there is no significant difference in terms of familiar object (X^2=6.791, p=0.079) and new object (X^2=5.651, p=0.130) and the total time they spent with both objects (X^2= 5.601, p=0.133) and Recognition Index (X^2 =7.125, p=0.068). As a result of the Mann Whitney U analysis ferformed to understand the source of the difference in terms of Discrimination Index; female animals in the AM group got significantly higher scores (0.59±0.34), than than female animals in the control (-0.03±0.37, Z=2.132, p<0.05), mild stress (-0.24±0.22, Z=-2.309, p<0.05) and severe stress (0.1±0.43, Z=-1.970, p<0.05) groups.

Long Term Memory

In the evaluation conducted without any gender discrimination; in the test for long-term memory, there was a significant difference between groups in terms of the time they spent on the new object (X^2=8.710, p<0.05). There was no significant difference found between the groups in terms of Familiar object (X^2=5.759, p=0.124) and the total time spent with both objects (X^2=7.655, p=0.054), Discrimination Index (X^2=7.216, p=0.065), and Recognition Index (X^2=6.819, p=0.078). It is found that the result of the analysis performed to understand the source of the difference in terms of the time they spent with the New object indicated that animals in the control group (13.17±12.78) spent significantly more time with new objects than the animals in severe stress (2.25±3.02, Z=-2.501, p<0.05) and AM (2.75±3.47, Z=-2.243, p<0.05) groups.

As a result of the analysis for male animals, while there was a significant difference between the groups in terms of Separation Index (X^2=8.220, p<0.05) and Recognition Index (X^2=8.666, p<0.05), there was no significant difference in terms of time spent with familiar object (X^2=5.589, p=0.133), New object (X^2=4.529, p=0.210) and total time spent with both objects (X^2=4.713, p=0.194). As a result of Mann Whitney U analysis forformed to understand the source of the difference in terms of Discrimination Index, it is found that the male animals in the mild stress group (0.73±0.33) got significantly higher scores than the animals in the control (0.17±0.24, Z=-2.262, p<0.05), severe stress (0.22±0.47, Z=-1.967, p<0.05) and ambivalent mother (0.07±0.25, Z=-2.560, p<0.05) groups. Similarly, in terms of Recognition Index, male animals in the Mild stress group (0.87±0.16) got significantly higher scores than the animals in the control (0.5±0.27, Z=-2.262, p<0.05), severe stress (0.51± 0.36,Z=-1.967, p<0.05) and ambivalent mother (0.48±0.23, Z=-2.786, p<0.01) groups.

Table 2. Gender comparison between groups

Variables	Groups (N / Column percentage)				X^2	p
Gender	Control	Mild stress	Severe stress	Ambivalent mother		
Male	6 (50%)	6 (50%)	5 (35.7)	8 (61.5)	1.816	0.611
Female	6 (50%)	6 (50%)	9 (64.3)	5 (38.5)		

No significant difference was found between the groups in terms of gender distribution (X^2= 1.816, p =.611).

Table 3. Comparison of ELISA test results for NMDAR2B protein levels of the groups (ng/mL), *p<0.001

Variables	Groups (Mean ± Standard error)				X^2	p
	Control	Mild stress	Severe stress	Ambivalent mother		
Prefrontal cortex	1.61±0.5	1.9±0.49	1.62±0.44	1.89±0.47	4.219	0.239
The hippocampus	1.88±0.42	4.65±1.71	3.36±1.08	4.57±1.41	31.416*	0.000

In the evaluation made for female animals; there was no significant difference found between the times spent with familiar object (X^2=5.954, p=0.114) and new object (X^2=6.624, p=0.085) and total time spent with both objects (X^2=6.669, p=0.083), Separation Index (X^2=1.092, p=0.779) and Recognition Index (X^2=1.688, p=0.640).

Social Interaction Test

In the evaluation of social behavior performance, conducted without gender discrimination; there was a significant difference between the groups in terms of social behavior (X^2=12.505, p<0.01) and stress behavior (X^2=13.508, p<0.01). There was no significant difference found between the groups in terms of Activity (X^2=6.634, p=0.085) and aggressive behavior (X^2=6.936, p=0.074). As a result of the analysis performed to understand the source of the difference in terms of social behavior, animals in the Mild stress group (13.56±18.08) showed significantly less social behavior performance than animals in the control (36.75±33.56, Z=-2.310, p<0.05) group and animals in the AM group (5.14±5.56) showed significantly less social behavior performance than animals in both the control (36.75±33.56, Z=-3.466, p<0.01) and the severe stress (21.19±22.90, Z=-2.034, p<0.05) groups. Animals in the control group (7.75±8.18) showed significantly less stress behavior performance than the animals in mild stress (23.68±16, Z=-3.167, p<0.01), severe stress (34.12±27.08, Z=3.292, p<0.01) and AM (30.38±23.78, Z=-2.369, p<0.05) groups.

As a result of the analysis performed for male animals, while there was no significant difference between the groups in terms of their social behavior (X^2=2.967, p=0.397) and stress behavior (X^2=2.264, p=0.519), there was a significant difference found between the groups in terms of activity (X^2=11.986, p<0.01) and aggressive behavior (X^2=11.079, p=0.05). As a result of the analysis performed to understand the source of the difference in activity, male animals in the severe stress group (3.23±3.97) showed significantly less activity performance than male animals in control (28.33±14.7, Z=-2.745, p<0.01), mild stress (23.89±14.46, Z=-2.745, p<0.01) and ambivalent mother (18.79±10.83, Z=-2.684, p<0.01) groups. Male animals in the Ambivalent mother group (0.24±0.23) showed significantly more aggressive behavior than the animals in the control (0±0, Z=-2.102, p<0.05), mild stress (0±0, Z=-2.102, p<0.05) groups.

In the evaluation made for female animals, a significant difference was found between the groups in terms of their social behavior (X^2=9.370, p<0.05) and stress behavior (X^2=14.818, p<0.01) performance. There was no significant difference between the groups in terms of activity (X^2=1.963, p=0.580) and aggressive behavior (X^2=1.739, p=0.628). As a result of the analysis performed to understand the source of the difference, the female animals in the control group (49.94±40.59) showed significantly more social behavior performance than female animals in mild stress (10.53±17.07, Z=-2.085, p<0.05) and AM (2±1.84, Z=-2.739, p<0.01) groups. In terms of stress behavior performance, it was observed that female animals in the control group (3.22±2.34) showed significantly less stress behavior performance than female animals in the mild stress (23.17±21.13, Z=2.882, p<0.01), severe stress (32.89±18.53 Z=-3.182, p<0.01) and AM (38.23±24.24, Z=-2.739, p<0.01) groups.

ELISA

Results of analysis of NMDAR2B protein levels obtained from prefrontal cortex and hippocampus of rats by ELISA (Enzyme-Linked ImmunoSorbent Assay) method for all groups are presented in Table 3. According to these results, a significantly higher NR2B level was found in the hippocampus brain region of the mild stress, severe stress and ambivalent mother groups compared to the control group; and in the ambivalent mother group compared to the severe stress group (X^2=31.416, p<0.001).

The results of the analysis of NMDAR2B protein levels obtained from the prefrontal cortex and hippocampus of male animals by ELISA method are presented in Table 4. According to these results, a significantly higher NR2B level in the hippocampus brain region was found in the mild stress, severe stress and ambivalent mother groups compared to the control group; and in the ambivalent mother group compared to the severe stress group (X^2= 18.986, p<0.001).

Finally, the results of the analysis of NMDAR2B protein levels obtained from the prefrontal cortex and hippocampus of female animals by ELISA method are presented in Table 5. According to these results, the female animals in the severe stress group were found to be significantly lower in the prefrontal cortex brain region than the female animals in both the mild stress and the ambivalent mother groups (X^2=7.869, p<0.05). Mild stress, severe stress and ambivalent mother groups were found to be significantly higher in the hippocampus brain region than the control group (X^2=12.503, p<0.01).

DISCUSSION

Environmental conditions have a significant impact on brain development, neuroplasticity and behavior. Exposure to various stressors in early life is an increased risk cause for mental illnesses that can occur in every period of life. Separating the newborn rat pups from the mother in the early period is the most used intervention method in scientific studies with rats. The aim of this study was to investigate whether there were behavioral differences between the rat pups whose mothers did not feed and give care the rat pups from time to time although they were with their offspring and rats

Table 4. ELISA test results for NMDAR2B protein levels of male groups *p <.001

Variables	Groups (Mean ± Standard error)				X^2	p
	Control	Mild stress	Severe stress	Ambivalent mother		
Prefrontal cortex	1.5±0.51	1.84±0.56	1.79±0.54	1.81±0.5	1.429	0.699
The hippocampus	1.8±0.45	4.68±1.71	2.99±0.58	5.07±1.3	18.986*	0.000

Table 5. ELISA test results for NMDAR2B protein levels of female groups *p<.05, **p, 01

Variables	Groups (Mean ± Standard error)				X^2	p
	Control	Mild stress	Severe stress	Ambivalent mother		
Prefrontal cortex	1.73±0.49	2.05±0.29	1.48±0.31	2.12±0.28	7.869*	0.049
The hippocampus	1.97±0.39	4.62±1.96	3.61±1.32	3.45±0.99	12.503**	0.006

separated from the mother in adulthood. In this context, we investigated the effect of created manipulation on stress response, learning/memory, social interaction type and expression of NMDA receptor sub-type NR2B on the hippocampus and prefrontal cortex.

In order to evaluate anxiety-like behaviors, the relevated t-maze, which is the most commonly used behavior test in researches, was utilized. Many studies have shown that rats leaving the mother exhibit more anxious behaviors due to restricted maternal care, and therefore spend less time in the open arms of the elevated t-maze (19). While the Ambivalent mother group rats and rats separated from the mother are expected to spend less time in the open arms compared to the control group, supporting the anxiety behavior; the data was that there was no statistically significant difference between the groups in terms of time spent in closed and open arms. However, as in this study, in some studies with rats leaving the mother in the early postpartum period, it was found that the elevated t-maze performances of the rats that were separated from the mother were not different from the control group rats, even if the daily time of separation from the mother was longer (20).

There are divergent data in the literature regarding the effect of early negative life events on performance in the new object recognition test. Cognitive function, which is measured as discrimination of the new object, has been depicted to be unaffected or impaired in MS groups (21).

Our data and findings also support the idea that separation from the mother does not affect recognition memory when evaluated regardless of gender. However, the relatively short duration of separation from the mother (1 hour) may cause memory not to be affected. Because there are studies in the literature showing that recognition memory is impaired when the time of separation from the mother is longer (3 hours) (22). However, when the genders were evaluated independently, there were differences in recognition memory performance between the gender groups. In the short-term recognition memory evaluation, it was found that females of the ambivalent mother group could distinguish the new object better from the familiar object, while mild stress group male rats had better performance in recognizing and discriminating the new object in the long-term recognition memory assessment compared to the other group rats.

In order to evaluate the effects of early interventions on the social interaction of rats in adulthood, a social interaction test was performed. In this behavioral experiment, the performance of rats (3 different types of behavior, namely social behavior, stress-related behavior and aggression), as well as the effect of gender on performances, were evaluated. When the social interaction test is evaluated without any gender discrimination, it was found that animals in the mild stress group showed less social behavior performance than the control group, and animals in the ambivalent mother group showed less social behavior performance than the control group and severe stress group. In addition, all of the manipulation groups showed to behave more stressed than the control group. When the male rats were evaluated alone, animals in the severe stress group showed less activity than other groups. Whereas the animals in the ambivalent mother group were found to be more aggressive than the animals in the control and mild stress group, they did not differ from the severe stress group in terms of aggressive behavior. As far as the female rats were compared in terms of the social interaction test performance between the groups, whereas the control group female animals showed more social behavior than the females in the mild stress and ambivalent mothers group, there was no difference found with the severe stress group. Again, females in the control group were found to depict less stress-related behavior compared to females in the intervention groups. Previous studies indicate that while exposure to manipulations that may cause stress decreases social interaction test performances (23), interventions with environmental enrichment result in decreased agonistic social interaction, increased aggression and an increase in recognition memory function in the new object recognition test in rodents (24). When the behavior experiment data is evaluated, the plush rat used in the ambivalent mother model may have become an enriching object in the cage environment. Exposure to this enriching object may have caused different behavioral effects in female and male rats.

Studies have shown that NR2B sub-units are selectively expressed in adult cerebral cortical and hippocampal stimulating neurons and that the expression decreased in the brain region with the onset of sexual maturity and aging (25). This seems to be consistent with the general view that some forms of learning and memory functions begin to show signs of decline when an animal enters the reproductive phase. Therefore, NR2B is ideal for manipulating the random detection properties of the NMDA receptor (26).

The increase in NR2B expression in the forebrain improves learning and memory function in the adult brain (27). Furthermore, in more anxious rats, the expression of NR2B subunit controlling emotional behavior increased in the frontal cortex and limbic structures. In this study, it was found that, when NMDA receptor subtype NR2B levels are examined, the NR2B levels of females of mild stress and ambivalent mother group were higher than the severe stress group, when the groups were evaluated in general, NR2B level was significantly higher in the hippocampus brain region in all manipulation groups compared to the control group, and that prefrontal cortex NR2B levels did not differ between groups. When the studies in the literature are examined, it is seen that the changes in the prefrontal cortex and hippocampus in adulthood after exposure to early separation from the NR2B subunit are inconsistent. There are studies indicating that NR2B expression increases in the prefrontal cortex and hippocampus or (29), while the prefrontal cortex level does not change; it increases only in the hippocampus (12).

While there is a study finding that the expression of the NR2B sub-unit in the hippocampus increases in males while decreases in females in the adult period after exposure to environmental enrichment, the number of studies on the effect of early environmental enrichment exposure on NR2B expression is limited (30). In addition to other findings, this study supports that early manipulations are particularly effective on the hippocampus NR2B level.

CONCLUSION

In order to create an ambivalent mother model, a plush toy rat in the same size as the actual mother was placed after the rats left the mother. The aim in doing so was, based on the presence of behavioral effects of the rat in the absence of the mother in the adult period, to determine whether the rat pups would differ from the rats that were separated from the mother in terms of emotional, cognitive and social communication features. Contrary to expectations, as far as the data is evaluated, the presence of plush toy mother may be considered as an intervention in terms of environmental enrichment. In addition, the relatively short separation period from the mother, and the number of female and male rats obtained from a limited number of mothers that are included in the study due to the ethical committee principles make it difficult to interpret the study results.

We could not repeat our study with higher number of rats for ethical reasons, but future studies including seperated groups of male and female rats with implementation of longer duration of seperation can increase the validity and reliability of the results.

Ethics Committee Approval: The Ethics approval for this study was obtained from the Gazi University Animal Experiments Local Ethics Committee with the number GU-ET-14069.

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