

# Environmental Enrichment Ameliorates Repetitive Behaviors in a Rat Model of Autism

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## Abstract

Adverse environmental experiences during early life identified as potential concerns for neurodevelopmental disorders including autism spectrum disorder (ASD) with 1% prevalence among populations. Maternal separation (MS) is an animal model that is widely used to study long-term behavioral abnormality. To date, a great deal of studies is focused on the potential therapeutic role of environmental enrichment (EE) for the early life stress consequences inducing anxiety, depression and learning deficits. However, the influence of sex on the effect of environmental enrichment for the autistic-like behaviors induced by maternal separation has not been studied. The focus of the current study is to investigate the therapeutic effect of environmental enrichment on the behavioral deficits related to ASD in female Wistar rats. Pups were separated from the mothers for 3 hours daily from PND1 to PND14. After weaning time (PND21), the rats were subjected to environmental enrichment until behavioral tests day. On PND42-PND50 autism-related behaviors such as social interaction, stereotype behaviors, anxiety behavior, and locomotion were tested. The results showed that EE reduced stereotype behaviors in maternal separation rat model of autism but increased anxiety behavior ( $p < 0.05$ ). Social behavior and locomotion decreased by maternal separation ( $p < 0.05$ ) but EE had no significant effect on these behavioral abnormalities ( $p > 0.05$ ). Current study demonstrates that repetitive behaviors induced by maternal separation can be treated by EE but the anxiety exacerbation may occur which needs more

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## 1. Introduction

There is increasing evidence that adverse early life experiences play a significant role in causing psychological disorders [1]. Brain development is a complex interactive process that depends not only on genes, but also on environmental factors and social interactions [2]. For example disruption in normal child-parent interactions as an early adverse experience may alter the essential inputs needed for brain development [3]. The quality of parent-infant relationship in infancy and childhood can affect different aspects of the child's development, including the level of the cognitive functioning, language, emotions, and social skills [4]. Human studies, for example, have shown that excessive use of digital devices in childhood can lead to impaired brain development by reducing the child's interaction with the environment, specially the mother [5]. Animal studies have also shown that separation from the mother during the first days of life leads to abnormalities in brain plasticity and behavioral disorders such as anxiety, depression, memory loss and impaired social behavior in adulthood [6-8]. It is noteworthy that in recent years the role of these adverse environmental experiences early in life has been demonstrated in the development of neurodevelopmental disorders such as autism which previously thought to be highly genetic [9].

Autism spectrum disorder (ASD) includes neurodevelopmental disorders with complex pathophysiology which are characterized by impaired social interactions and stereotyped behaviors [10]. In recent years, the prevalence of ASD in the world has increased dramatically, reaching 1% of the population [10]. ASD is now considered as a multifactorial disorder caused by genetic and non-genetic risk factors and their interaction [11]. To find the pathophysiology and effective treatments for ASD, animal studies are of high importance. Different animal models especially rodent ones of this disorder have been used [12]. Recent studies have shown that maternal separation (MS) during breast feeding in rats can induce autistic like behaviors and therefore highlighted the effect of this negative experience in developing ASD [9, 13].

Rodents in laboratory are generally reared in groups of four animals per cage with accessing only to food and water. Environmental enrichment (EE) is a paradigm in which the animals are reared in large cages with different objects and play equipment and more number of animals to get more sensory inputs and social interactions to improve the cognitive performance [14]. This paradigm is used in different animal models as a treatment and positive effects of it has been reported [15].

Accordingly, in this study, we aimed to study the effects of environmental enrichment on the autism associated behavioral disorders induced by maternal separation in female rats which has not been studied up to now.

## 2. Material and methods

In this study, 9 adult male and female rats (10-12 weeks old) were used for mating and 32 female pups were randomly selected for the experiment. The rats were kept in standard conditions with 12 /12 light-dark cycle (lights on at 7:00 AM), 21 °C controlled temperature and free access to food and water. The experiment consists of four groups: 1. MS 2. MS.EE 3. Control 4. Control.EE. Pups of groups 1 and 2 were separated from the mother 3 hours daily (9am- 12am) from PND1 to PND14. In control groups, the pups were kept with their mother until weaning (PND21).

### *Environmental Enrichment*

After weaning on PND21, EE was applied for the groups 2 and 4. For enriching the environment rats were kept in big cages with different objects, tunnels and playing tools, with more number of rats (n=8-9 in each cage). While in untreated groups (1 and 3), rats reared in normal condition with n=4 rat in each cage until behavioral tests day.

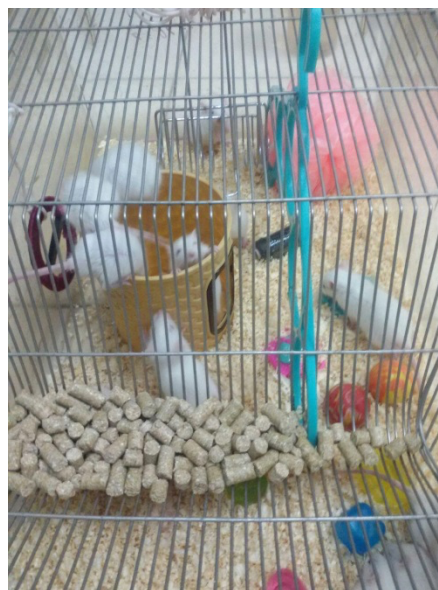


Figure1. In treatment groups rats were exposed to environmental enrichment from PND21-PND42. During this time the objects and play tools were changed every 5 days.

### *Behavioral Tests*

On PND42-PND50 behavioral tests were done at 8am-3pm. Autism related behavioral tests including three-chamber test, open field and marble burying tests were taken for examining social behavior, stereotype, anxiety and locomotive behaviors. To perform the behavioral tests, rats were transferred to the behavioral testing room one hour before the test. All tests were performed for each rat in one day and rested for one hour between trials. All tests were video-taped with the DVR system. Test boxes were rinsed with 70% alcohol before each test.

### ***Three-chamber Test***

In this test, briefly the subject rat was placed in a three-chamber box. The middle chamber was empty and in one of the two chambers, there was a strange rat inside a small cage and in the other side, there was only an empty cage. The rat had 10 minutes to freely explore the chambers and then the time spent in the strange rat chamber and time spent in the empty cage chamber were recorded as sociability behavior.

### ***Marble burying Test***

In this test, 20 spherical marbles were arranged on the surface of the bedding in 5 rows. The test rat was kept in the box for 30 minutes and after the 30 minutes, the number of marbles seized by the rat was counted. The criterion for counting was 75% of the marbles covered with bedding which is considered as a stereotype behavior.

### ***Open field Test***

In the open field test, a box divided into lines and one central circle was used. The rat was placed in the box for 10 minutes and the following parameters were recorded:

1. Time spent in the central circle. Rats with higher anxiety spend less time in the central circle.
2. Time spent for self-grooming, which was recorded as another measure of repetitive behavior.

### ***Statistical Analysis***

All statistical analyzes were performed by SPSS software version 22. Q-Q plot was used to check the normality of the data distribution. Leven's test was used to test the equality of variances. Within group comparisons were performed using repeated measures ANOVA. And for all other behavioral tests, two way ANOVA and Tukey post-hoc test were used. Logarithmic transformation was performed on the original data of self-grooming due to inequality of variance level. Data are presented as mean  $\pm$  SEM. The null hypothesis was rejected with  $p < 0.05$  as the level of significance.

## **3. Results**

### ***Repetitive Behaviors***

In this study, two tests were used to investigate repetitive behavior. Time spent for self-grooming in the open-field test and numbers of buried marbles in the marble burying test were considered as repetitive behaviors in rodents. The analysis of the marble burying test (Fig 2A) data shows that MS group buried significantly more number of marbles in comparison with both control groups ( $p < 0.001$ ). And MS.EE group buried significantly less number of marbles than the MS group ( $p < 0.001$ ). Self-grooming data analysis also shows (Fig 2B) that MS group spent significantly more time for self-grooming than the all other three groups ( $p < 0.001$ ). There was no significant difference between other groups ( $p > 0.05$ ). It should be noted that due to inequality of variance level logarithmic transformation was performed on the original data of this test.

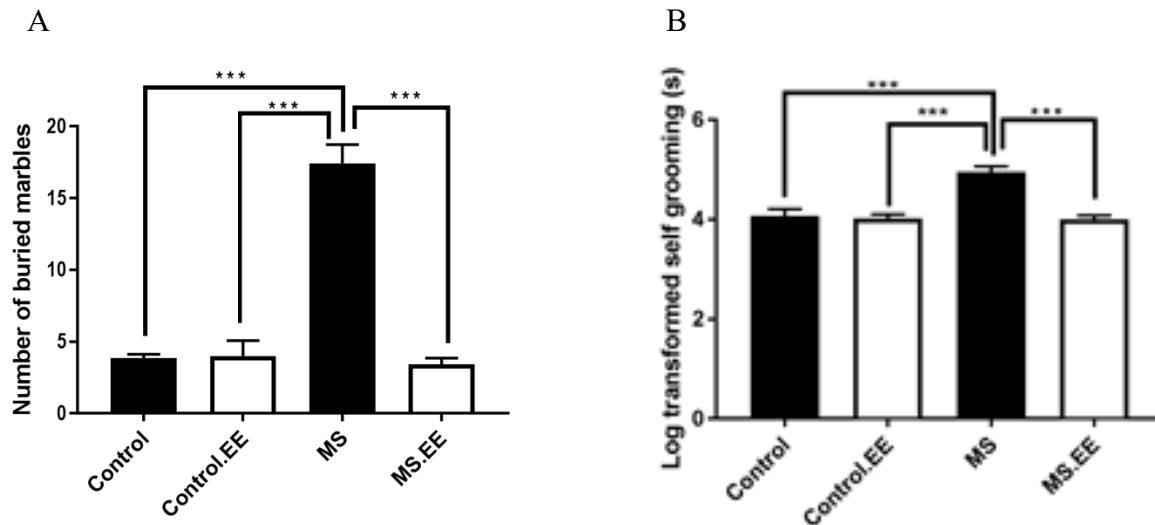


Figure 2. MS and EE effects on adolescent female rats repetitive behaviors: A) Marble burying and B) Self-grooming. \*\*\* $p < 0.001$ . Original data of self-grooming is log transformed due to inequality of variance.

### Social Behavior

Within group comparisons with repeated measures ANOVA in the three-chamber test showed that both MS and MS.EE groups spent less time in the stranger rat chamber than the empty cage chamber ( $p < 0.001$ ). While in the control and control.EE groups, rats spent significantly more time in the stranger rat chamber than the empty cage chamber ( $p < 0.001$  and  $p < 0.008$ ) (Fig 3). This means that maternal separated rats were less likely to engage in social interaction and preferred to spend more time in the empty cage chamber.

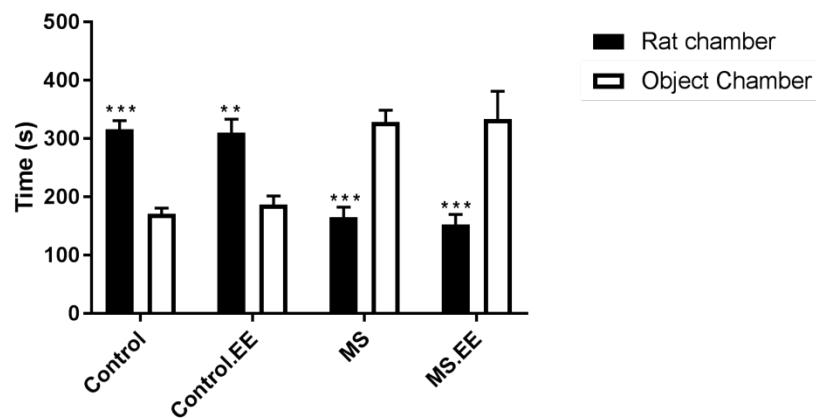


Figure 3. MS and EE effects on adolescent female rat's social behavior. Stars indicate the significant difference between time spent in strange rat chamber and empty cage chamber within each group. \*\* $p < 0.01$ , \*\*\* $p < 0.0001$

### Anxiety Behavior

The duration of the rat presence in the central circle of the open-field box was recorded to investigate the anxiety behavior. The less time spent in this circle indicates more anxiety. In this test, the results of two-way ANOVA and Tukey post-hoc analysis showed that control group spent significantly more time in the central circle than the other three groups ( $p < 0.001$ ). Indicating that maternal separation and environmental enrichment both have led to increased anxiety behavior. There was no significant difference between other groups ( $p > 0.05$ ) (Fig 4).

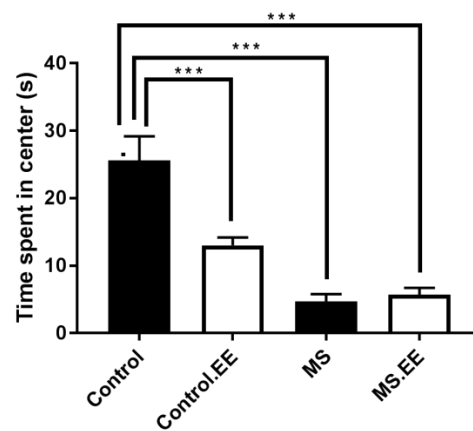


Figure 4. MS and EE effects on adolescent female rats anxiety behavior. The less time spent in the center arena of open field indicates more anxiety. \*\*\* $p < 0.001$

### Locomotion

Another parameter recorded in open field test was the number of lines crossed by rat as a locomotion behavior. As seen in Fig 5, this parameter was significantly less in MS and MS.EE groups in comparison with control group ( $p = 0.002$  and  $p = 0.003$ ). There was no significant difference between other groups ( $p > 0.05$ ).

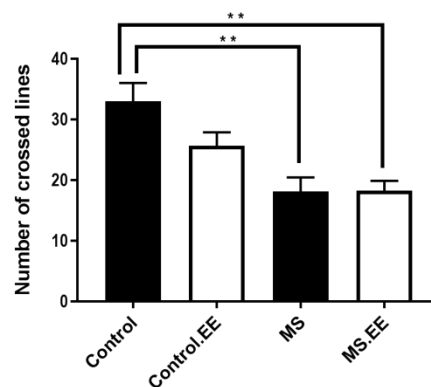


Figure 5. MS and EE effects on adolescent female rats locomotion behavior. \*\* $p < 0.01$

#### 4. Discussion

In the current study, maternal separation induced autistic like behaviors (impaired social and repetitive behaviors) and increased anxiety behavior along with reduction in locomotion in adolescent female rats. More importantly, the repetitive behaviors ameliorated by environmental enrichment. We detected no effect of EE on the social behavior deficit and locomotion and on the other hand, EE elevated the anxiety behavior.

The physiology and development of infants are regulated by mother-infant interactions [16, 17]. Therefore, daily separation in the first few days after birth is a negative experience for the infant and leads to behavioral changes later in life with impaired brain plasticity [18]. A study in 2014 reported for the first time the effect of maternal separation on the development of an animal model of autism in rats with the improvement of these symptoms with lithium injection [9]. Several other studies subsequently confirmed this model [13, 19]. Given that many genes are implicated in autism, this model can be much more applicable than genetic models, and it clearly demonstrates the important role of environmental factors in the development of neurodevelopmental disorders such as autism, which has previously been highly genetically conceived [20]. Therefore higher anxiety and autistic like behaviors induced by MS in our work is consistent with those previous studies [21]. Reduction in locomotion behavior in maternal separated rats was unexpected since this parameter was recorded to examine the hyperactivity behavior as comorbid symptom in autistic children. However this could be due to the higher anxiety or depression which has been shown in maternal separated rodents in previous studies [22]. Human studies also have shown that isolation from mother can have long lasting effects on the behaviours such as anxiety and depression [23, 24]. In 2016, Berg et al. conducted the first study on the rate of adverse childhood experiences in children with autism and stated that ASD diagnosis is significantly associated with the experience of childhood stressful events. They suggested that children and adults exposed to stress-related phenomena are at the risk of mental disorders. Pelphrey, et al considered disturbances in the early development of the brain regions with the social function deprive the infant brain from social developmental opportunities [25]. These findings suggest that ASD is not an unpreventable and incurable disorder. As Dawson says, symptoms of autism, with a focus on mother-child interaction, are treatable and even preventable [26].

Although it is difficult to mimic the behavioral therapies in animals but to find the mechanism of this intervention, using animal models is of importance especially in the ASD, because currently the only effective treatment for this disorder is behavioral intervention [27]. Environmental enrichment has been long used in animal models of developmental disorders and the positive results have been reported but there is some contrary evidence and the mechanism is not very clear [28-30]. In this study, we found that environmental enrichment could ameliorate repetitive behaviors in maternal separated female rats. Previous studies suggest that this effect may be through different ways in CNS including spine density, dendritic branching and even angiogenesis [31]. Surprising result in our work is that EE exacerbated the anxiety behavior in maternal separated and control animals. Although many studies have shown the reduced anxiety by EE [32], this is not the first time finding negative effect of EE on anxiety behavior. For example effects of prenatal EE on male rats indicated that EE offspring showed more anxiety-like

behaviors and less activity, compared to controls [33]. More over in 2018 adverse effect of EE on the anxiety and social behavior in a genetic animal model of autism have been demonstrated [28]. These contradictory results can be due to different parameters like genetic background and enrichment methods which is different in laboratories. In this work EE was applied after weaning, this sudden change in the environment may be stressful itself increasing anxiety behavior in animals. Accordingly we suggest that in future studies the EE be applied from the beginning of the pup's life. But the important note is the need for more detailed research to find the mechanism and answer the open questions in this regard.

## 5. Conclusion

Taken together, this study demonstrates the improvement of repetitive behaviors in maternal separation animal model of autism but exacerbation of the anxiety behavior. Suggesting further investigations before translating it to human.

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## Conflict of interest

No competing financial interests exist.

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