Original Article

# Sex-dependent effects of postweaning exposure to an enriched environment on visceral pain and anxiety- and depression-like behaviors induced by neonatal maternal separation

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**Background:** Neonatal maternal separation (NMS) can lead to visceral pain and anxiety- and depression-like behaviors. An enriched environment (EE) can alleviate NMS-induced pain and mental disorders, but previous studies have mostly been performed in male animals. Therefore, the aim of this study was to investigate whether the effects of EE were sex dependent at different stages of development.

**Methods:** Female and Male C57BL/6 J mice that had been subjected to NMS alone and those subjected to both NMS and exposed to EE were used in this study. The visceral pain threshold test (PTT), open field test (OFT), sucrose preference test (SPT), and forced swimming test (FST) were conducted to evaluate visceral pain, anxiety-like behavior, and depression-like behavior in mice, respectively.

**Results:** Compared with the male mice in the NMS group without EE exposure, those exposed to EE from postnatal day (P)21 to 41 showed an increase of the visceral pain threshold in the PTT, an increase of the central time and central distance in the OFT, an increase of the sucrose preference rate in the SPT, and a decrease of the time of immobility in the FST. Compared with both female and male mice in the NMS group without EE exposure, those exposed to EE from P21 to P61 had an increase of the visceral pain threshold in the PTT, an increase of the central time and central distance in the OFT, an increase in the sucrose preference rate in the SPT, and a decrease of the time of immobility in the FST.

**Conclusions:** EE is more effective in male NMS mice, while longer EE is required in female NMS mice for positive effects.

**Keywords:** Enriched environment (EE); neonatal maternal separation (NMS); visceral pain; anxiety- and depression-like behaviors; sex differences

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## Introduction

Chronic visceral pain is a very common disease, often accompanied by mental disorders such as anxiety and depression, which seriously affects the quality of life of patients and exacts a heavy economic burden on patients and society. At present, its treatment mainly relies on drug therapy, which is limited by poor compliance, significant side effects, and unsatisfactory treatment effects mainly due

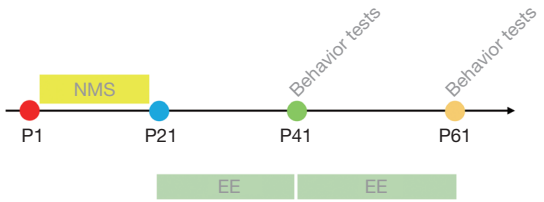


**Figure 1** Timeline of experiment. Testing the effects of EE exposure (P21–P41 and P21–P61) on visceral pain and anxiety- and depression-like behaviors in the female and male NMS mice. Behavior tests: the PTT, OFT, SPT, and FST. EE, enriched environment; NMS, neonatal maternal separation; PTT, pain threshold test; OFT, open field test; SPT, sucrose preference test; FST, forced swimming test; P, postnatal day.

to unclear etiology and pathogenesis (1). Recent studies have shown that early life stress affects brain development and predisposes individuals to chronic pain and psychiatric disorders (2,3). For example, neonatal maternal separation (NMS) prolongs stress-induced hypothalamic-pituitary-adrenal axis (HPA axis) responses, affects synaptic plasticity and brain anatomy, reduces function in areas such as the prefrontal cortex and hippocampus (4), and increases the risk of chronic pain and psychiatric disorders (5,6).

A growing body of research suggests that a rich environment can promote brain development in a number of ways. An enriched environment (EE) is an environmental intervention frequently used in animal research to improve quality of life as its name implies, and it is defined as a combination of "complex inanimate objects and social stimuli" designed to increase freedom of movement, sensory (including auditory, visual, olfactory, tactile, etc.) and social stimuli (7). Noxious stress is known to cause dysfunction of the HPA axis, which is associated with visceral pain and psychiatric symptoms. In contrast, an EE improves chronic pain and psychiatric disorders caused by early life stress, including visceral pain, anxiety, and depression (8). Several studies have shown that an EE can reduce anxiety- and depressive-like behaviors in animals (9,10). Moreover, an EE can reduce the expression level of plasma corticosterone (11,12). Our previous research demonstrated that EE exposure can reduce NMS-induced visceral pain as well as anxiety- and depression-like behaviors in adolescence and adulthood (8). Cumulatively, previous studies indicate that an EE can reduce visceral pain and anxiety- and depression-like behaviors induced by NMS (8-10). However, these studies have been mainly performed on male animals, and thus we wanted to explore whether the role of EE exposure in NMS mice is sex related.

In this study, we used the NMS model to assay the effects of EE exposure from postnatal day (P)21 to 41 and P21 to P61 on the visceral pain threshold in the pain threshold test (PTT), center time and center distance in the open field test (OFT), the sucrose preference rate in the sucrose preference test (SPT), and the time of immobility in the forced swimming test (FST) in female and male mice. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-476/rc).

## Methods

### *Animals*

Pregnant female mice were purchased from the Experimental Animal Center of Xuzhou Medical University. Mice were placed in a constant temperature and humidity environment with a 12-h light-dark cycle with free access to water and food. Mice were killed immediately if they developed disease. After behavioral tests, the mice were killed. All experimental procedures were approved by the Animal Care and Use Committee of Xuzhou Medical University (No. 202010A132), in compliance with the national guidelines for the care and use of animals. and the study protocol was prepared before the study but was not registered.

### *Neonatal maternal separation*

From P1 to P21, neonatal mice were kept away from their mother for 6 h every day. After weaning at P21, female and male mice were used for subsequent experiments.

### *Enriched environment*

EE cages were enriched with various running wheels, climbing platforms, toys, and pipes. Interior items and colors were rearranged and updated weekly.

### *Experimental design*

According to the random number table method (*Figure 1*), 16 male and 16 female mice were blindly and randomly divided into 2 groups (n=8 mice per group), respectively: an NMS group and an NMS + EE group. In the NMS group, from P1 to P21, neonatal mice were separated from their mothers for 6 h every day until weaning at P21; in the NMS

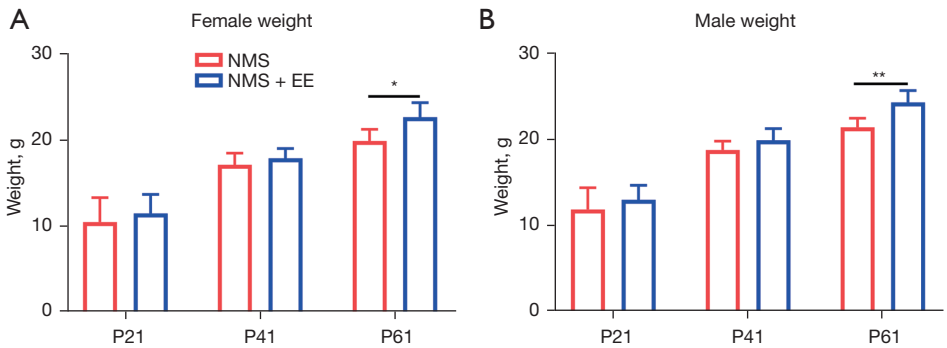


**Figure 2** The effects of adolescent EE exposure (P21–P61) on body weight in (A) female and (B) male mice. All data are expressed as mean ± SD. *, P<0.05, **, P<0.01. NMS, neonatal maternal separation; EE, enriched environment; P, postnatal day.

+ EE group, from P1 to P21, neonatal mice were separated from their mothers for 6 h per day until weaning at P21 and then placed in the EE from P21 to P61. At P41 and P61, mice were subjected to behavioral tests. All researchers who performed the animal experiments and analyzed the data were blinded to the treatment conditions.

### *Visceral pain threshold test*

After the mice were anesthetized, an uninflated balloon was placed in the colorectum. After a moment of adaptation, the balloon was inflated to give pressure to the intestinal wall, and the pressure value when the abdominal wall muscle left the platform was considered to be the visceral pain threshold.

### *Open field test*

The device was divided into 9 squares equally. The area in the middle was called the central area. The mouse was gently placed in the central area, and mouse behavior was recorded for 5 min. Observation indicators included total distance, center time, and center distance.

### *Sucrose preference test*

Mice were housed in single cages and trained on sucrose drinking water for 48 h. Two bottles of 1% to 2% sucrose water were given in the first 24 h. In the second 24 h, one bottle was 1% to 2% sucrose water while the other bottle was pure water (the positions of the 2 bottles were exchanged halfway). After the mice were fasted for 14 to 23 h, the sucrose water preference index was measured as the amount of water (g) that the mice drank from 2 bottles of water within 1 h. Sucrose preference was calculated as the following evaluation index: sucrose preference (%) = sucrose water consumption/(sucrose water consumption + drinking water consumption) × 100%.

### *Forced swimming test*

The mice were placed in glass tanks (diameter: 11 cm; height: 25 cm), which were filled with tap water at 21–24 ℃. The mice were forced to swim for 6 min, and the time of immobility during the last 4 min was measured as an indicator of depression-like behavior.

### *Statistical analysis*

All data are expressed as mean ±SD. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Comparisons between multiple groups were performed by 2-way analysis of variance (ANOVA) followed by a post hoc Bonferroni test. A P value less than 0.05 was considered statistically significant.

## Results

### *EE exposure from P21 to P61 increased the body weight of adult female and male NMS mice*

To investigate the effect of EE on weight gain, mice were weighed daily between P21and P 61 (*Figure 2*). The results showed that initial body weight (P21) and body weight in mice exposed to 20 days of the EE (P41) did not differ from those in the NMS group in both female and male mice; however, the body weight in both female and male mice

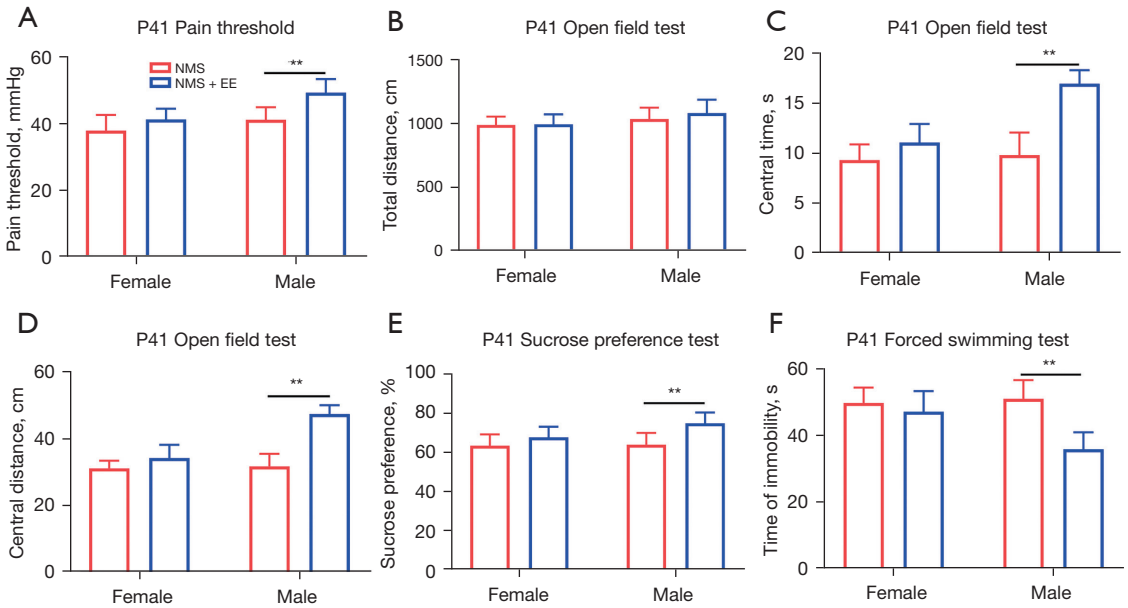


**Figure 3** The effects of EE exposure (P21–P41) on visceral pain and anxiety- and depression-like behaviors in the female and male NMS mice. The (A) visceral pain threshold in the PPT; (B) total distance, (C) central time, and (D) central distance in the OFT; (E) sucrose preference in the SPT; and (F) time of immobility in the FST of female and male mice were measured in all experimental groups. All data are expressed as mean ± SD. **, P<0.01. EE, enriched environment; NMS, neonatal maternal separation; PTT, pain threshold test; OFT, open field test; SPT, sucrose preference test; FST, forced swimming test; P, postnatal day.

after 40 days of EE exposure (P61) increased.

### *EE exposure from P21 to P41 alleviated NMS-induced visceral pain and anxiety- and depression-like behaviors in male mice*

In the comparison of the female mice in the NMS group *vs.* the NMS + EE group, the visceral pain threshold in the PTT, central time and central distance in the OFT, sucrose preference rate in the SPT, and time of immobility in the FST did not change significantly after 20 days of EE exposure (*Figure 3*). In contrast to this, compared with the male mice in the NMS group, the male mice in the NMS +EE group showed an increase of visceral pain threshold in the PTT, an increase of central time and central distance in the OFT, an increase of sucrose preference rate in the SPT, and a decrease of time of immobility in the FST (*Figure 3*).

### *EE exposure from P21 to P61 alleviated NMS-induced visceral pain and anxiety- and depression-like behaviors in female and male mice*

Compared with the NMS group, the NMS + EE group had an increase of visceral pain threshold in the PTT, an increase of central time and central distance in the OFT, an increase of sucrose preference rate in the SPT, and a decrease of time of immobility in the FST in both female and male mice after 20 days of EE exposure from P21 to P61 (*Figure 4*).

## Discussion

In this study, we used the NMS model to assay the effects of EE exposure from P21 to P41 and from P21 to P61 on the visceral pain threshold in the PTT, center time and center distance in the OFT, sucrose preference rate in the SPT, and time of immobility in the FST in female and male mice. EE exposure from P21 to P41 caused an increase in the visceral pain threshold in the PTT, an increase of central time and central distance in the OFT, an increase of the sucrose preference rate in the SPT, and a decrease of time of immobility in the FST only in male mice. EE exposure from P21 to P61 caused an increase of visceral pain threshold in the PTT, an increase of central time and central distance in the OFT, an increase of the sucrose preference rate in the SPT, and a decrease of the time of

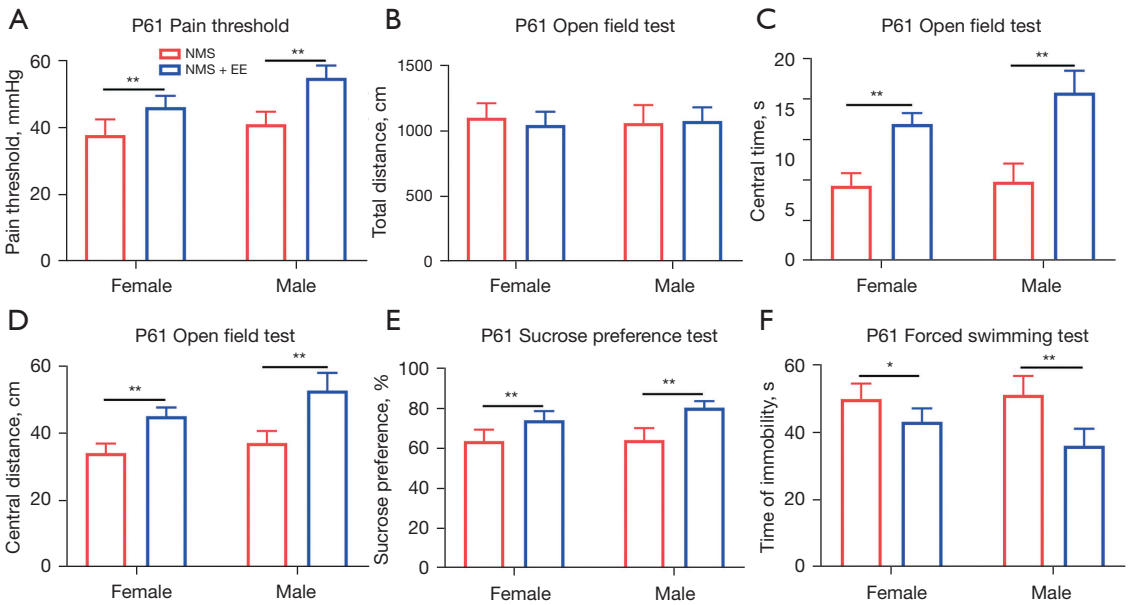


**Figure 4** The effects of EE exposure (P21–P61) on visceral pain and anxiety and depression-like behaviors in the female and male NMS mice. (A) The visceral pain threshold in the PPT; (B) total distance, (C) central time, and (D) central distance in the OFT; (E) sucrose preference in the SPT; and (F) time of immobility in the FST of female and male mice were measured in all experimental groups. All data are expressed as mean ± SD. *, P<0.05, **, P<0.01. EE, enriched environment; NMS, neonatal maternal separation; OFT, open field test; SPT, sucrose preference test; FST, forced swimming test.

immobility in the FST in both female and male mice. Our study suggests that EE is more effective in male NMS mice and that longer EE exposure is required in female NMS mice for positive effects.

Numerous studies have shown that early life adversity can severely impair brain development, reduce synaptic plasticity, negatively affect behavior, and contribute to the development of chronic pain, depression, and anxiety (2,5-7,13). The most commonly used method to improve NMS-induced pain, anxiety- and depression-like behaviors was medication, but it has many side effects, poor efficacy, poor compliance, and high cost. In contrast, EE has relatively low cost, no side effects and good compliance.

The application of EE, originally derived from neurorehabilitation, had been shown to be effective in conditions such as brain injury, improving functional recovery, anxiety and depression (14-16). Because EE provided a stress-free environment, promoted multiple sensory experiences, and enhanced social interactions, it may have advantages over routine coercive activities for pain management. Anatomical changes in the central nervous system caused by chronic pain can lead to mental disorders such as depression and anxiety (17). In patients with brain injury, EE prevents hippocampal atrophy for several years (18). Therefore, we hope that EE may ameliorate subsequent anxiety and depression by ameliorating pain-induced neuroanatomical changes.

Our previous study found that NMS caused a decreased pain threshold and anxiety- and depression-like behaviors at P41 and P61, while EE exposure could alleviate the negative effects caused by NMS in male mice (8). However, EE exposure in female mice has not been sufficiently examined. In this study, we found that NMS-induced visceral pain and anxiety- and depression-like behaviors improved in both male and female mice after 40 days of EE exposure (P61), suggesting that EE alleviates early life stress damage regardless of sex. However, after 20 days of EE exposure (P41), there was no positive effect on female mice, while the effects on male mice were significant. These results mean that female mice are less sensitive to EE than are male mice.

In contrast to the consistent results concerning the effect of an EE in male animals, there are few and contradictory studies on the effects of an EE in females. One study reported that EE exposure increased sensitivity to noxious stimulation (19), while another found that EE exposure did not affect sensitivity to nociceptive stimuli (20). In our

study, short EE exposure (P21–P41) had no significant effect on NMS-induced visceral pain or anxiety- and depression-like behaviors, while longer EE exposure (P21–P61) significantly improved the negative effects caused by NMS, which may provide a reasonable explanation for the discrepancies of previous studies (19,20). Our results suggest that female animals are less sensitive to an EE and require longer EE exposure to reverse the negative effects of early life stress. Given that there is evidence that women are more sensitive to pain than are men, primarily due to sex hormones (21), the difference in EE exposure between women and men may be related to estrogen. Besides sex dependence, it was found that EE at different stages of development had different effects on NMS-induced visceral pain and anxiety- and depression-like behaviors, so we need to further explore the differences in EE intervention between female and male mice at different developmental stages.

In conclusion, a short period of EE exposure before adulthood ameliorates NMS-induced visceral pain and anxiety- and depression-like behaviors in male mice, while a longer period of EE exposure has a positive effect on female NMS mice. This may have implications for EE exposure in guiding the clinical treatment of visceral pain, anxiety, and depression caused by early life stress.

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## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-22-476/rc

*Data Sharing Statement:* Available at https://tp.amegroups.com/article/view/10.21037/tp-22-476/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-22-476/coif). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures were approved by the Animal Care and Use Committee of Xuzhou Medical University (No. 202010A132), in compliance with the national guidelines for the care and use of animals.



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