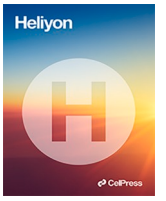
Heliyon 10 (2024) e27037

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

A single 24 h maternal separation at PND 9 promotes behavioral resilience of female C57BL/6J mice and the possible role of hippocampal Homer1a

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ARTICLE INFO

Keywords:
Early life stress (ELS)
Maternal separation (MS)
Behavioral resilience
Hippocampus
Homer1a

ABSTRACT

Early life stress (ELS) has been thought to increase vulnerability to developing psychiatric disorders later in life, while some researchers have found that adversity early in life may promote stress resilience. Studies investigating the resilient effect of maternal separation (MS) are still relatively few, and the underlying mechanisms remain unknown. In the current study, the effect of a single 24 h MS paradigm at postnatal day 9 (PND 9) in female C57BL/6J mice was investigated by assessing behavioral performance in middle adolescence. We demonstrated that, mice in MS group displayed decreased anxiety-like behavior and increased exploratory behavior than controls in the open field test and elevated plus maze test. Furthermore, MS mice exhibited improved hippocampal-dependent spatial learning in the Morris water maze test. This performance indicated behavioral resilience to early life stress. The protein expression levels of Homer1 isoforms, which are implicated in a variety of neuropsychiatric disorders, were evaluated using Western blot analysis. A significant increase in hippocampal Homer1a protein expression was observed immediately after MS, which subsequently decreased until adolescence (PND 27–42), when a significant increase was observed again. This distinctive change of hippocampal Homer1a protein expression pattern indicated that hippocampal Homer1a might play a role in behavioral resilience to MS in female C57BL/6J mice. In conclusion, this study demonstrated that exposure to a single 24 h MS at PND 9 promoted behavioral resilience of female C57BL/6J mice in middle

Abbreviations: ELS, early life stress; MS, maternal separation; CON, control; PND, postnatal day; SHRP, stress hyporesponsive period; PSD, postsynaptic density; OF, open field; EPM, elevated plus maze; MWM, Morris water maze; BCA, bicinchoninic acid; ANOVA, analysis of variance; SEM, standard error of mean; NMDA, N-methyl-D-aspartate; mGluR5, metabotropic glutamate receptor 5.

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https://doi.org/10.1016/j.heliyon.2024.e27037
Received 1 November 2023; Received in revised form 22 February 2024; Accepted 22 February 2024
Available online 23 February 2024

adolescence. This behavioral resilience might be related to increased expression of hippocampal Homer1a.

1. Introduction

Early life experience plays an essential role in mental development [1]. Many studies in humans and animals have demonstrated that ELS increases vulnerability to developing psychiatric disorders later in life [2–5]. Maternal separation for 3 h daily from PND 2 to 14 in mice was found to provoke anxiety-like behaviors [6]. Another study using the same MS paradigm demonstrated that MS significantly increased the immobility time in the forced swimming test, indicating more depression-like behavior [7]. However, some researchers have found that adversity early in life may promote stress resilience in later life [8–10]. Exposure to moderate stressors during childhood contributed to an attenuated depressive response to proximal negative events during adolescence [11]. Rodent models of MS are widely used to study behavioral responses to early life stress and the underlying mechanisms [12]. Predictable maternal separation was found to promote stress resilience in rats [9]. Mice offspring who experienced brief maternal separation in early life showed resilience to chronic stress in adulthood [13]. This discrepancy in MS research may be attributable to experimental design, strain differences, frequency, and predictability. Understanding the mechanisms underlying such resilience may shed light on preventive methods for stress susceptibility [9]. However, studies investigating the resilient effect of maternal separation are still relatively few, and the underlying mechanisms remain unknown. There is a clear need to further explore such resilience and the underlying mechanisms.

Previous studies have suggested that C57BL/6 mice exhibited more resilience to MS during the early postnatal period compared with other strains [10,14,15]. Exposure to a brief MS early in life increased the subsequent resilience of male C57BL/6 mice to the effects of social stress on vulnerability to cocaine [16]. C57BL/6J offspring exposed to MS exhibited decreased anxiety and unchanged stress-induced corticosterone response as adults, indicating resilience [10]. Although psychiatric disorders appear more prevalent in females than in males, there are relatively fewer studies on females than males including animal research [17]. Past research has mostly focused on the effects of MS on male offspring; few studies have focused on the effects on female offspring [18]. Our former research found that female rats were more resilient to early life stress than males [19]. More research focused on females are needed. A review proposed that the specific time window between postnatal day 9 (PND 9) and PND 10 is the most critical for the outcomes of MS [20]. Therefore, in this study, we intended to further explore the possibility that exposure to a single 24 h MS at PND 9 might promote behavioral resilience of female C57BL/6J mice in middle adolescence.

Homer1 is a scaffolding protein located in the postsynaptic density (PSD) and exists in two major splice variants, Homer1a and Homer1b/c [21]. The longer and constitutively expressed variant, Homer1b/c, consists of a conserved amino-terminal target-binding domain and a coiled-coil structure that allows multimerization. The short isoform Homer1a, which lacks the coiled-coil structure, acts as an immediate early gene product, and is normally induced by synaptic activation [22]. Homer1 isoforms are involved in synaptic plasticity [23], and are implicated in a variety of neuropsychiatric disorders, such as depression, schizophrenia, and stress-related disorders [24,25]. The relationship between Homer1 and stress has been investigated in animals, including acute stress [26], chronic social stress [27], and restraint stress [28]. However, to the best of our knowledge, little research has investigated the relationship between Homer1 and early life stress [23,29]. And the findings seemed to raise the possibility that Homer1 might be implicated in the mechanism of resilience to early life stress. The hippocampus plays an important role in cognition, emotional regulation, and stress response [13]. In this study, we aimed to investigate the possible role of hippocampal Homer1 in behavioral resilience to MS in female C57BL/6J mice.

2. Materials and methods

2.1. Animals

Pregnant C57BL/6J mice were obtained from the Laboratory Animal Center of the Fourth Military Medical University, Xi'an, China. All mice were housed under standard laboratory conditions with 12-h light/dark cycle (lights on at 08:00 a.m.), and controlled temperature (21 ± 1 °C) and relative humidity (55 ± 5%). Mice were administered food and water *ad libitum*. All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University (XJYYLL-2015251) and were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. The MS paradigm

A single 24 h MS paradigm was established to model early life stress. The pregnant C57BL/6J mice were single-housed in plastic cages and checked twice daily at 9:00 and 17:00 for parturition. For each litter, the date of birth was designated as PND 0. One day after birth, litters were culled to 6 pups (3 males and 3 females), and litters were randomly assigned to either a maternal separation (MS) group or control (CON) group. On PND 9 at 9:00 a.m., the MS pups were separated from their dams and were singly placed for 24 h in isolation cages filled with bedding from their home cages [30]. The isolation cages were placed in a different room to the dams to prevent olfactory or vocal communication, and the cages were placed on heating pads maintained at 31 ± 1 °C. After 24 h, the MS pups

were returned to their home cages and were rolled in the soiled home-cage bedding before being reunited with the dams. The pups of the control groups were not disturbed during this time. Following the separation procedure, animals were left undisturbed except for routine cage cleaning. On PND 21, the sexes of the pups were confirmed, and pups were weaned and group-housed in same-sex caging for 6 mice in one cage. The male pups were used in a separate experiment and were not included in this study. A total of 105 female offspring were used in this study. The experimental timeline is outlined in Fig. 1.

2.3. Behavioral tests

Mice from the CON group (n = 11) and the MS group (n = 10) were subjected to a battery of behavioral tests between PND 42 and PND 47 in the following sequence [31,32]: open field test (OF), elevated plus maze test (EPM), and Morris water maze test (MWM). Tests began from 8:00 a.m. each day. Only one female pup per litter was used in each group to avoid a litter effect [33].

2.3.1. Open field test

The open field test was performed in a square wooden arena (50 × 50 cm) with a wall height of 40 cm, and the area was divided into 16 quadrants (4 central and 12 peripheral area). To minimize stress, mice were transported to the testing room at least 1 h prior to the experiments to allow for acclimatization. During the one-session test, each mouse was placed in the center of the arena and was allowed to explore freely for 15 min. Behavior was recorded and analyzed using the Ethovision video-tracking system (Noldus, Netherlands). The arena was cleaned with alcohol solution after each test. The percentage of time spent and percentage of distance moved in the center area were used as indexes of anxiety, while the total distance moved was used to evaluate exploration.

2.3.2. Elevated plus maze test

The elevated plus maze was shaped like a plus sign and consisted of two opposite open arms (30 × 5 × 15 cm) and two opposite closed arms (30 × 5 × 15 cm) extending out from a central platform (5 × 5 cm). The mice were placed individually in the center of the maze facing one of the open arms, and were allowed to explore the maze freely for 5 min. The behavior of each mouse was recorded and analyzed using the Ethovision video-tracking system (Noldus, Netherlands). The maze was cleaned with alcohol solution after each test. The percentage of open arm entries, percentage of distance moved in the open arms, and percentage of time spent in the open arms were used as anxiety measures, while the number of total entries into open and closed arms was used as exploration measures.

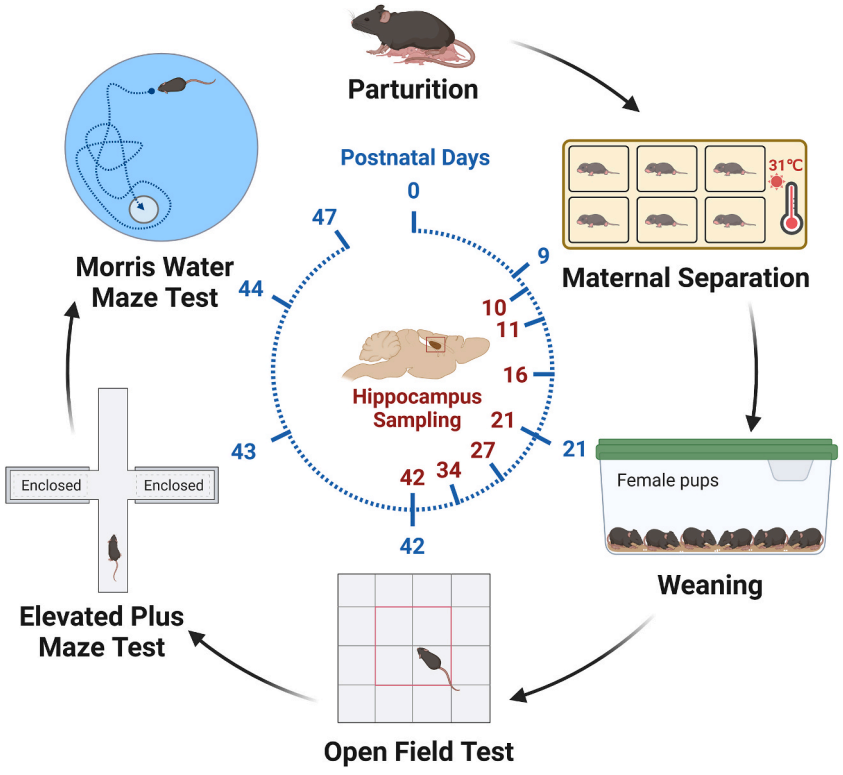

Fig. 1. Experimental timeline depicting parturition, maternal separation, weaning, behavioral testing, and hippocampus tissue sampling. Created with BioRender.com.

2.3.3. Morris water maze test

The Morris water maze consisted of a large circular tank (diameter, 120 cm; depth, 40 cm) filled with water made opaque by the addition of milk. The water temperature was maintained at 26 °C. An escape circular platform (diameter, 9 cm) was submerged 1.5 cm below the water surface. Several visual cues were placed on the walls of the testing room as spatial references. The performance of the mice was recorded using the Ethovision video-tracking system (Noldus, Netherlands). Mice were subjected to a spatial acquisition training consisting of four trials/day for 4 consecutive days. During each trial, the platform was placed in the center of one of the four quadrants of the MWM, termed quadrant Q, which corresponded to the same location for all mice. The mice were released individually into the water facing the wall of the tank in a randomly selected quadrant (any of the four quadrants, except quadrant Q), and were allowed to swim until they reached the platform or for a maximum of 60 s. The animals were manually guided to the platform if they failed to locate it. At the end of each trial, mice were allowed to stay on the platform for 30 s before being removed, towel-dried, and returned to their cages. The latency to find the platform during spatial acquisition training was used to evaluate hippocampal-dependent spatial learning and memory.

2.4. Western blot analysis

Mice hippocampus tissue sampling was performed at seven time points, namely PND 10, 11, 16, 21, 27, 34 and 42. At 9:00 a.m. of the aforementioned PNDs, mice (n = 6 in each group per time point) were weighed, and subsequently were sacrificed by rapid decapitation after anesthesia. For each time point, only one female pup per litter was used in each group to avoid a litter effect [33]. The bilateral hippocampi were immediately dissected as described previously [34] and frozen in liquid nitrogen. The samples were homogenized at 4 °C using a Whole Cell Lysis Assay kit (KeyGEN BioTECH, Nanjing, China). The protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Jiangsu, China). Denaturation was performed by boiling the samples for 5 min. Subsequently, after adding sample buffer to aliquots containing 30 μg protein, the samples were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). Then, the membranes were incubated for 1 h in blocking buffer (10 mM Tris, 100 mM NaCl, and 5% non-fat dried milk), followed by incubation with primary antibody at 4 °C overnight. The primary antibodies were rabbit anti-Homer1a antibody (1:500, Synaptic Systems, Goettingen, Germany), rabbit anti-Homer1b/c antibody (1:1000, Synaptic Systems, Goettingen, Germany), or mouse anti-β-actin antibody (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). The immunoreactive bands were imaged using an image analysis system (Fotodyne, Hartland, WI, USA) and their optical density was quantified using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA, USA). To ensure that equal amounts of protein were loaded onto each lane of the gel, β-actin was used as an internal standard.

2.5. Statistical analysis

Data were analyzed using SPSS Statistics 25 (IBM Corporation, Armonk, NY, USA). Behavioral tests data, except for the latency in the MWM test, was analyzed using the Student's *t*-test. Repeated measures analysis of variance (ANOVA) was used to analyze the group difference in the latency of the MWM test. For body weights and Western blots data, Student's *t*-test was used to analyze the group difference between CON and MS at each time point. Statistical significance was set at $p < 0.05$. Graphs were plotted using GraphPad Prism 10 (GraphPad, La Jolla, CA, USA).

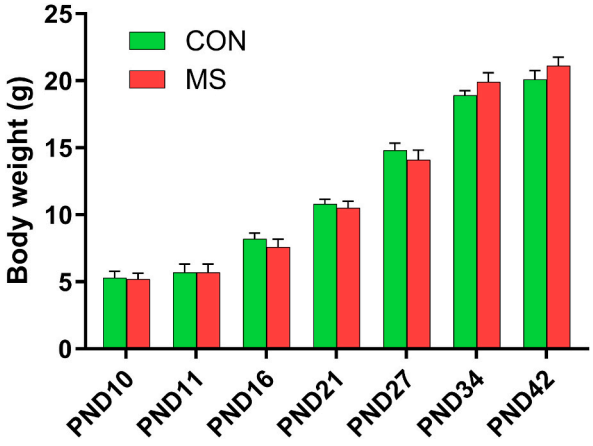

Fig. 2. Effect of MS on body weight gain in female C57BL/6 offspring mice. Body weight was measured on PND 10, 11, 16, 21, 27, 34 and 42 before the sacrifice to obtain hippocampus tissue. Values are mean ± SEM of 6 mice per group. CON, control; MS, maternal separation; PND, postnatal day.

3. Results

3.1. Body weight gain was not affected by MS

To investigate the effect of 24 h MS on the body weight gain in female C57BL/6J offsprings, the body weight of pups was measured on PND 10, 11, 16, 21, 27, 34 and 42 before the sacrifice to obtain hippocampus tissue. As illustrated in Fig. 2, pups in both CON and MS group exhibited a significant continuous increase in body weight, but no significant group difference between CON and MS group was found (all $p > 0.05$), indicating the body weight gain was not affected by MS.

3.2. Decreased anxiety and increased exploration after MS

To assess the effect of MS on anxiety-like and exploratory behaviors, the mice were subjected to the OF test (Fig. 3) and the EPM test

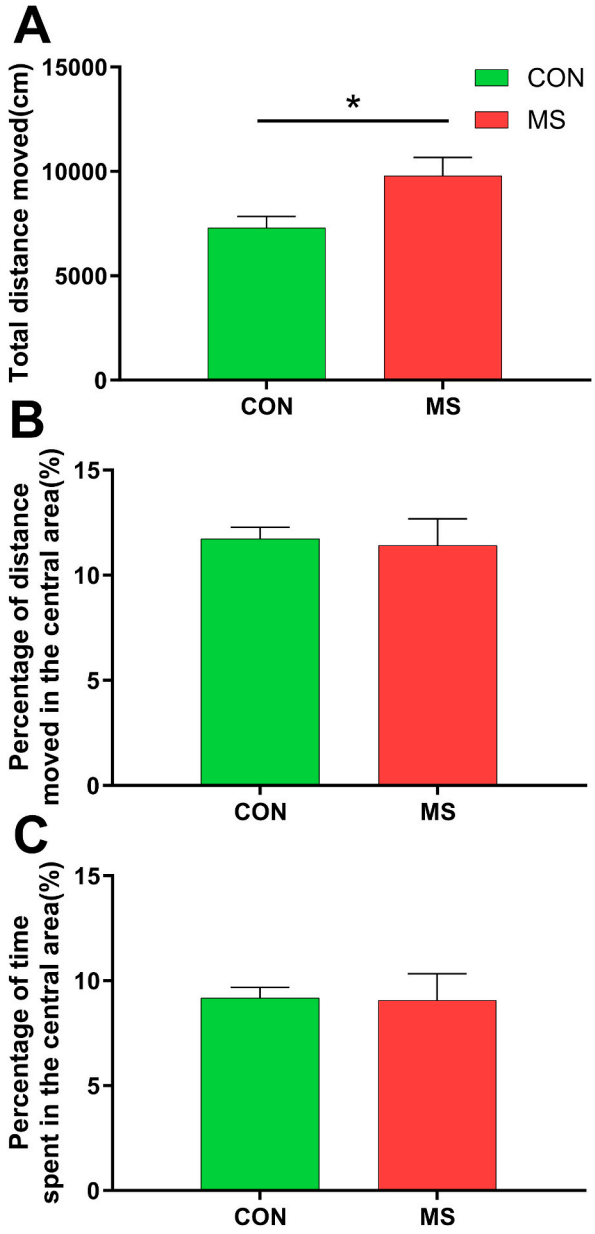

Fig. 3. Effect of MS on anxiety-like and exploratory behaviors in the open field test on PND 42. Comparisons between CON (n = 11) and MS (n = 10) mice. (A) Total distance moved. (B) Percentage of distance moved in the center area. (C) Percentage of time spent in the center area. Values are mean ± SEM of all mice per group. CON, control; MS, maternal separation; PND, postnatal day. *$p < 0.05$.

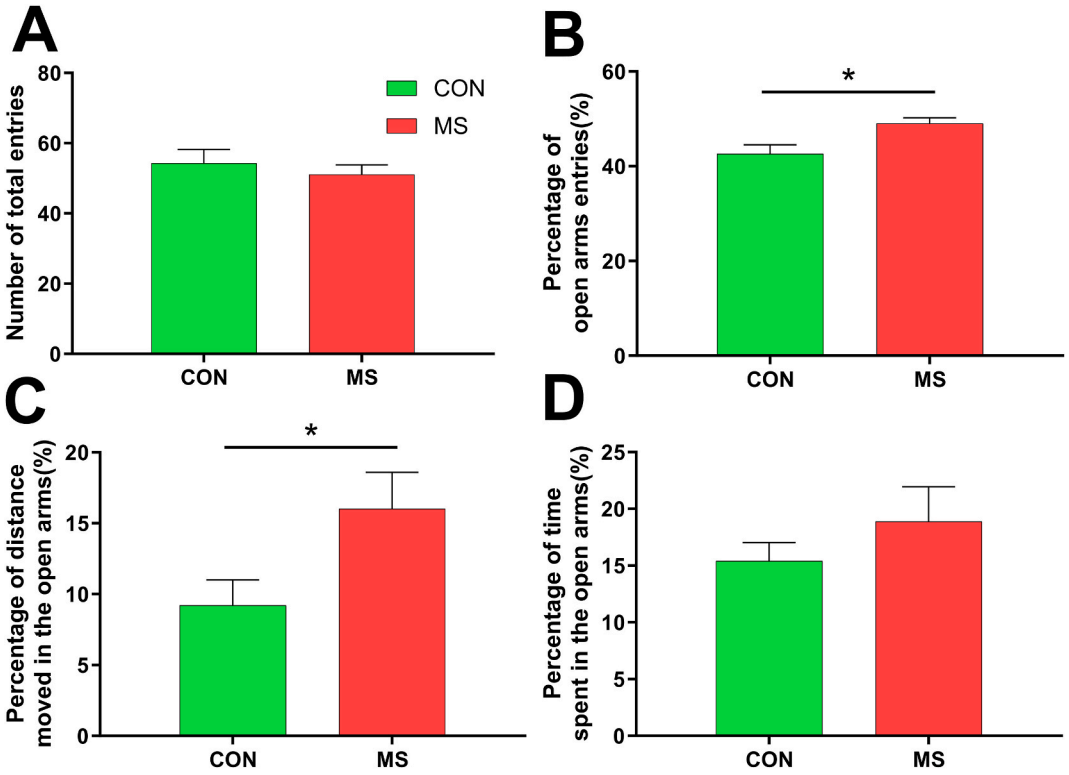

Fig. 4. Effect of MS on anxiety-like and exploratory behaviors in the elevated plus maze test on PND 43. Comparisons between CON (n = 11) and MS (n = 10) mice. (A) Number of total entries (number of entries into the open and closed arms). (B) Percentage of open arm entries. (C) Percentage of distance moved in the open arms. (D) Percentage of time spent in the open arms. Values are mean ± SEM. CON, control; MS, maternal separation; PND, postnatal day. *p < 0.05.

(Fig. 4). Compared with the CON group, the MS group covered overall a longer distance in the OF test (p = 0.024), indicating higher velocity of locomotion (Fig. 3(A)). Moreover, we found MS mice displayed a higher percentage of both open arm entries (p = 0.012) and distance moved in the EPM open arms (p = 0.041) (Fig. 4(BandC)). The mice in MS group also had a higher percentage of time spent in the EPM open arms, but the difference was not significant (p = 0.316) (Fig. 4(D)). These findings suggested that mice in MS group displayed decreased anxiety and increased exploration. However, no differences were observed between CON and MS group in the percentage of both time spent (p = 0.939) and distance moved (p = 0.818) in the center area of the open field (Fig. 3(BandC)). And no differences were observed between the MS and CON group in the number of total entries in the EPM test (p = 0.523) (Fig. 4(A)).

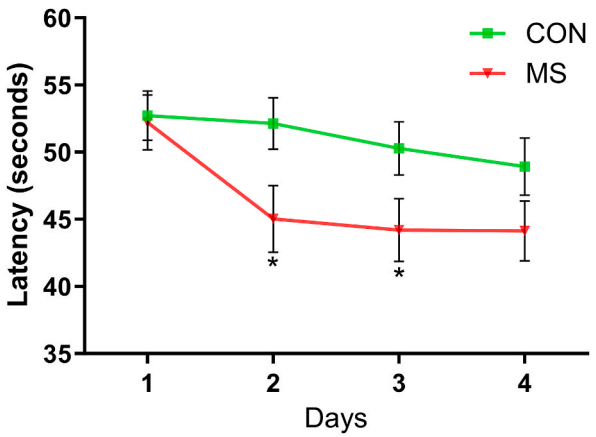

Fig. 5. Effect of MS on spatial learning and memory in the Morris water maze test on PND 44–48, indicated by latency to find the platform during spatial acquisition training (four trials). Comparisons between CON (n = 11) and MS (n = 10) mice. Values are mean ± SEM. CON, control; MS, maternal separation; PND, postnatal day. *p < 0.05.

3.3. Improved hippocampal-dependent spatial learning following MS

The effect of MS on hippocampal-dependent spatial learning and memory was evaluated by subjecting mice to the MWM test (Fig. 5). The repeated measures ANOVA for escape latency showed significant main effects of day and treatment (time: $F_{(3,82)} = 3.266$, $p = 0.022$, treatment: $F_{(1,82)} = 8.407$, $p = 0.005$), but not day × treatment interaction ($F_{(3,82)} = 0.978$, $p = 0.404$). Mice from the MS group exhibited a more rapid decrease in the latency to find the submerged platform during the spatial acquisition training compared with the CON group; the difference was statistically significant on training day 2 ($F_{(1,82)} = 5.230$, $p = 0.025$) and day 3 ($F_{(1,82)} = 3.977$, $p = 0.049$), indicating improved hippocampal-dependent spatial learning.

3.4. The pattern of hippocampal Homer1a protein expression changes following MS

Western blotting was used to assess the postnatal protein expression of Homer1 isoforms in the hippocampus of mice from CON and MS group (Fig. 6(A)). Compared with the CON group, mice from the MS group exhibited a distinctive pattern of Homer1a protein expression change. We observed that compared with the CON group, Homer1a protein expression of mice from the MS group was significantly up-regulated at PND 10, which was immediately after the maternal separation ($p < 0.001$), followed by a significant down-regulation at PND 11 and PND 16 (PND 11: $p = 0.025$; PND 16: $p < 0.001$) and then was significantly up-regulated again at PND 27, which lasted until PND 42 (PND 27: $p = 0.005$; PND 34: $p < 0.001$; PND 42: $p < 0.001$) (Fig. 6(C)). No group differences were observed in Homer1b/c protein expression at any time point, except that at PND 21, the expression of Homer1b/c protein was significantly lower in the MS group than in the CON group ($p = 0.043$) (Fig. 6(B)).

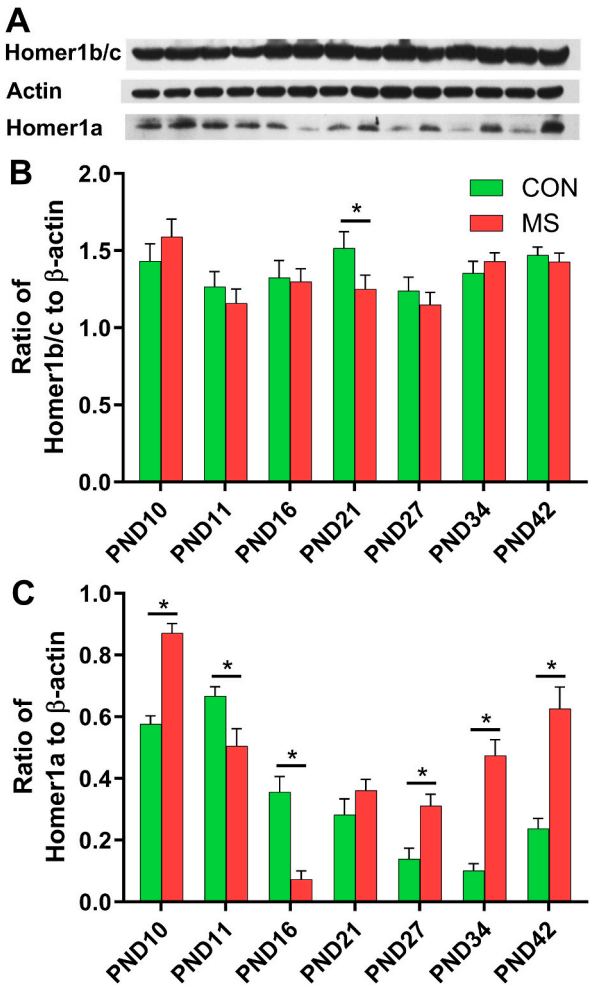

Fig. 6. Effect of MS on Homer1a and Homer1b/c protein expression levels in the hippocampus of female C57BL/6 mice evaluated on PND 10, 11, 16, 21, 27, 34 and 42. (A) Representative Western blots of Homer1b/c, β-actin, and Homer1a (Uncropped version of blots was provided as supplementary material). The order of the Western blot panels in (A) was the same as that in (B) and (C). Relative levels of Homer1b/c (B) and Homer1a (C). Values are mean ± SEM of 6 mice per group. CON, control; MS, maternal separation; PND, postnatal day. *$p < 0.05$.

4. Discussion

In this study, we demonstrated that a single 24 h MS at PND 9 resulted in reduction in anxiety-like behaviors and improvement in spatial learning in female C57BL/6J mice during middle adolescence, indicating behavioral resilience to early life stress. Mice exposed to MS displayed decreased anxiety-like behaviors and increased exploration in the OF and EPM tests. Improved hippocampal-dependent spatial learning in the MWM test was also observed following MS. In addition, the distinctive pattern of hippocampal Homer1a protein expression changes following MS indicated that hippocampal Homer1a might play a role in behavioral resilience to MS in female C57BL/6J mice.

Previous studies demonstrated that MS provoked depressive- and anxiety-like behaviors in mice [6,7]. But there were studies suggesting that C57BL/6 mice exhibited more resilience to MS during the early postnatal period compared with other strains [10,14,15]. Both male and female C57BL/6J mice exhibited more resilience to stress than other strains, especially the stress-susceptible mouse strain BALB/c [35,36]. In the current study, we found that female mice in MS group displayed decreased anxiety-like behavior, increased exploratory behavior and improved hippocampal-dependent spatial learning.

Our findings of resilience to MS in female C57BL/6J mice are in agreement with previous studies. Brief MS was found to promote resilience to anxiety-like and depressive-like behaviors in female C57BL/6J offspring and reverse the damage of neuroplasticity injury [18]. Another study reported subtle decrease in anxiety and minor increase in activity in male C57BL/6J mice after MS [10]. Decreased anxiety in the light/dark box test and increased exploration in the EPM test were also reported among male C57BL/6J mice exposed to repeated MS from PND 1 to PND 14, but not in male BALB/c mice [14]. These previous studies mainly investigated the effect of repeated daily maternal separations. Under the predictable repeated MS paradigm, we could not rule out the possibility that pups might become habituated to the maternal absence and dams might adapt and provide compensatory care to the separated pups when reunited, which could potentially compensate for the negative effects of MS [10]. Therefore, we applied a single 24 h MS paradigm at PND 9 in the present study. Even so, we observed that female C57BL/6J mice still exhibited behavioral resilience.

Past research in rats reported long-lasting body weight reduction in both males and females following MS, which persisted into adolescence [37], early adulthood [38], or even late adulthood [39]. Such reductions might possibly be due to metabolism disruptions following MS, including a long-lasting decrease in leptin levels [40]. However, in C57BL/6J mice, the body weight gain was reported to decrease [4,14,41] or to remain unaffected [42] after MS. In our present study, the body weight gains of female C57BL/6J mice did not differ between the MS and CON groups. To some extent, this phenomenon could reflect the resilience of female C57BL/6J mice to early life stress.

Together, these findings confirmed the behavioral resilience of C57BL/6J mice following MS, but the underlying mechanisms remain unknown. Understanding the mechanisms underlying such resilience may shed light on preventive methods for stress susceptibility [9]. Recent studies seemed to raise the possibility that Homer1 might be implicated in the mechanism of resilience to early life stress [23,29]. Thus, we evaluated hippocampal Homer1 protein expression from PND 10 all along to PND 42. We observed that compared with the CON group, Homer1a protein expression of mice from the MS group was significantly up-regulated at PND 10, which was immediately after the maternal separation, followed by a significant down-regulation at PND 11 and PND 16, and then was significantly up-regulated again at PND 27, which lasted until PND 42. This distinctive pattern of hippocampal Homer1a protein expression changes following MS indicated that hippocampal Homer1a might play a role in behavioral resilience to MS in female C57BL/6J mice. This result was in line with recent reports demonstrating that hippocampal Homer1a in adult female mice was upregulated by repeated MS early in life [23]. Indeed, our findings indicated that increased Homer1a expression in the hippocampus of female adolescent C57BL/6J mice might be related to the behavioral resilience to MS. Further investigations are warranted to reveal the mechanism underlying these changes.

In contrast to the Homer1a findings, Homer1b/c exhibited relatively stable expression levels during the same period in both the CON and MS groups. It is well known that Homer1b/c is constitutively expressed in the hippocampus, while Homer1a acts as an immediate early gene product and is induced in an activity-dependent manner [22,43]. This might account for the unchanged expression level of Homer1b/c following MS paradigm.

Homer1 proteins have been implicated in the mechanism of resilience to stress. Homer1 acts as a moderator of the N-methyl-D-aspartate (NMDA)/metabotropic glutamate receptor 5 (mGluR5) complex, which is highly implicated in stress-related neuropsychiatric pathologies [44]. It was reported that increased expression of Homer1a in the hippocampus modulated the resilience to stress-induced depression [45]. Furthermore, disruption of Homer1a increased the vulnerability of mice to predictable subtle stress, suggesting that Homer1a might play a critical role in resilience to subtle stress [28].

Our present findings indicated a possible association between increased Homer1a expression in the hippocampus and behavioral resilience to MS in female adolescent C57BL/6J mice. While Homer1b/c links mGluR5 to the intracellular signaling machinery, Homer1a acts as a dominant negative isoform disrupting the GluR5/Homer1b/c coupling [27]. Thus, it might be inferred that increased Homer1a expression in MS mice exerted its effects by uncoupling GluR5/Homer1b/c. Further research is needed to elaborate the underlying mechanisms, and hence to seek preventive methods for stress susceptibility.

5. Conclusions

In the current study, we demonstrated that exposure to a single 24 h MS at PND 9 promoted behavioral resilience of female C57BL/6J mice in middle adolescence. This behavioral resilience might be related to increased expression of hippocampal Homer1a. Nevertheless, this plausible association warrants further research.

Ethics approval

All experimental procedures were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University (XJYYLL-2015251) and were conducted in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Funding

This work was supported by Wenzhou-Kean University International Faculty Research Support Program (IRSPK202201), Wenzhou-Kean University International Collaborative Research Program (ICRP2023007), National Natural Science Foundation of China (No.82304965), Provincial Science and Technology Youth Foundation of Gansu (22JR11RA007) and Scientific Research Project for Youth Cultivating of The 940 Hospital of PLA Joint Logistic Support Force (2021yxky067).

Data availability statement

The authors do not have permission to share data.

CRediT authorship contribution statement

Yelu Hao: Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Yujie Niu:** Visualization, Software. **Fei Shi:** Methodology, Formal analysis. **Lei Zhang:** Software. **Cheng Peng:** Methodology. **Zhiqiang Yan:** Methodology. **Xiaoyan Chen:** Methodology. **Hongyu Xu:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We wish to thank Dr. Han Song for his advice on the statistical analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27037.

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