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Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice

Authored by:

Qais Jarrar; Rami Ayoub; Kawther Alhussine; Khang Wen Goh; Said Moshawih; Chrismawan Ardianto;
Bey Hing Goh; Long Chiau Ming

Published in:

J. Pers. Med. 2022, Volume 12, Issue 11, 1921



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Status	Website online
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Publication Certificate	<input type="text" value="A4"/>
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Article type	Article
Title	Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice
Journal	<i>Journal of Personalized Medicine</i> (https://www.mdpi.com/journal/jpm)
Volume	12
Issue	11
Abstract	Background: The elevated plus maze (EPM) and the marble burying (MB) tests are common behavioral tests used for behavioral phenotyping in mouse models for neurodevelopmental disorders. However, the behavioral effects of maternal separation (MS), a standard paradigm for early life stress in animals, in both the EPM and MB tests remain incompletely known. Objectives: This study aimed to investigate the behavioral effects of prolonged MS in the offspring of mice using the EPM and MB tests. Methods: Male BALB/c mice were isolated from their mothers for 4 h each day during the first 30 days after birth. On day 50 postnatal, groups of separated and non-separated mice (<i>n</i> = 18/each group) were subjected to the EPM and MB tests for comparative behavioral evaluations. In addition, the locomotor activity of mice was evaluated using the actophotometer test. Results: The findings of the EPM test revealed that separated mice exhibited anxiolytic-like behaviors, as evidenced by a significant increase in the latency to closed arms and the time spent in the open arms compared with non-separated mice. Separated mice also showed compulsive burying activity in the MB test, as determined by a significant increase in the number of buried marbles. The results of the actophotometer test did not show any significant change in locomotor activity. Conclusions: Prolonged MS caused the adult offspring of mice to exhibit a decrease in anxiety state and increased compulsive burying activity, which were not associated with a change in locomotor activity. Further investigations with validated tests are needed to support these findings.
Keywords	maternal separation; anxiety; compulsivity; adaptation; reproductive health; pregnancy; marble burying test; mental disease; depression; impulsivity



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Manuscript Information

Received Date	1 September 2022
Revised Date	15 November 2022
Accepted Date	15 November 2022
Published Date	17 November 2022
Submission to First Decision (Days)	72
Submission to Publication (Days)	76
Round of Revision	3
Size of PDF	2505 KiB
Word Count	4849
Page Count	15
Figure Count	7
Table Count	0
Reference Count	102



Decision Accept in current form
Comments Nice Manuscript
Decision Date 15 November 2022

Review Report

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Journal JPM (https://www.mdpi.com/journal/jpm) (ISSN 2075-4426)

Manuscript ID jpm-1922536

Type Article

Title Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice (https://www.mdpi.com/2075-4426/12/11/1921)

Authors Qais Jarrar * , Rami Ayoub , Kawther Alhussine , Khang Wen Goh , Said Moshawih , Chrismawan Ardianto * , Bey Hing Goh , Long Chiau Ming

Abstract Background: The elevated plus maze (EPM) and the marbles burying (MB) tests are common behavioral tests used for behavioral phenotyping of mouse models for neurodevelopmental disorders. However, the behavioral effects of maternal separation (MS), a standard paradigm for early life stress maternal separation; anxiety; compulsivity; elevated plus maze; marbles burying test; psychopa-ty; impulsivity tests. Methods: Male BALB/c mice were isolated from their mothers for 4 hours each day during the first 30 days after birth. On day 50 postnatal, mice were subjected to the EPM, and MB tests for behavioral evaluations. In addition, the locomotor activity of mice was evaluated using the actophotometer test. Results: The findings of the EPM test revealed that separated mice exhibited anxiolytic-like behaviors, as evidenced by a significant increase in the latency to closed arms and the time spent in the open arms compared with non-separated mice. Separated mice also showed compulsive burying activity in the MB test, as determined by a significant increase in the number of buried marbles. The results of the actophotometer test did not show any significant change in locomotor activity. Conclusions: Prolonged MS caused adult offspring of mice to exhibit a decrease in anxiety state and increased compulsive burying activity, which were not associated with a change in locomotor activity. Further investigations with validated tests are needed to support these findings.

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Author's Notes

Reviewer 1

Comment 1: I do not understand how this study relates to JPM, a journal that aims at bringing all aspects of personalized medicine to one platform. The authors also do not discuss this.

The replies: The relationship of the current study with JPM is discussed in the revised manuscript as follows:

Over the past few decades, there has been mounting evidence that adverse childhood experiences can affect how individuals respond to both disease and treatment (Levy and Muench, 2022). Many studies showed that childhood adversity can cause epigenetic changes (Lundgaard Donovan et al., 2021) and increase individual's susceptibility to developing various illnesses such as cancer, heart disease, endocrine disorders and mental illnesses (Krause et al., 2020; Thumfart et al., 2021). Likewise, findings of the current study provide supporting evidence that mice exposed to early life stress by being separated from their mothers (from day 1-30 postnatal) were more likely to exhibit aberrant behaviors than their non-separated peers. These behaviors were characterized by significant decrease in anxiety state and significant increase in compulsive-like behaviors. Therefore, the maternal separation can influence the inter-individual variation in the susceptibility of diseases, such as anxiety and compulsive related disorders, and their pharmacotherapy.

Comment 2:

- The authors state that 'the behavioral effects of maternal separation (MS), ..., in both the EPM and MB tests remain incompletely known'. I do not understand how this study helps to shed light on this topic. Apart from several studies that investigated the effect of 'regular' periods of MS on anxiety, there are also some studies which investigated the effect of a prolonged period of MS (mainly about 20-21 days) on the EPM and other behavioral tests (e.g., Troakes & Ingram, 2008; meta-analysis by Wang et al., 2020, Transl Psychiatry for summary; ...).



The replies: Although most previous studies focused on the effects of maternal separation during infancy and the early weaning period (from day 1-21 postnatal), this study, however, was designed to examine the effect of a prolonged separation procedure that covered the prepubertal period (from day 1-30 postnatal). Research on brain development suggested that the maturing brain during prepubertal and adolescence period may be particularly vulnerable to the effect of stress, and that effect may significantly increase the risk of developing risky behaviors later in life (Winters and Arria, 2011) and cause differential impact on fear-related behaviors in a sex- and age specific way (Toledo-Rodriguez and Sandi, 2007). Similarly, this study showed an evidence that prolonged MS reduced the anxiety level which reflects a risky-taking behavior. In addition, this is the first study used to investigate the effect of prolonged maternal separation on the marble burying activity in the offspring of mice. We added this explanation in the revised manuscript.

- Moreover, in contrast to the current study, most of the other studies included additional analyses, such as cFos mRNA expression etc., to provide insights into the neuronal mechanisms. I am further missing a comparison to regular or shorter periods of MS in BALB/c mice.

The replies: We do appreciate the reviewer for raising this concern. Unfortunately, I am afraid that it is not possible to perform the requested analyses at the present time as animals were dissected and no more plasma, serum and tissue sample are available. This part of analysis will be considered in the future work. In response to the reviewer's comment, we addressed this issue and other unavoidable limitations in the revised manuscript as follows:

Although this study has reached its aims, there were some unavoidable limitations. First, this research was restricted to exploring the alterations in behavioral phenotypes. Therefore, further studies are needed to involve some molecular evaluations. Second, our results did not determine whether the behavioral alterations in this study were attributed to the effect of MS itself or whether they were indirectly related to the stress inflicted on the mothers that could affect the care given to their pups. This could be solved by evaluating measures related to maternal care. Finally, as the male and female mice were not separated in the current study, the behavioral tests were conducted only on the male mice to avoid concerns related to the effect of sex maturation and possible gestation.

-And what is the effect on female offspring? 'Interfering hormones' and 'data fluctuations' are not a good enough reason to leave these tests out. Furthermore, there seems to be a lot of variation within the non-separated males.

The replies: The reason for exclusion of female mice from the current study was clarified in the revised manuscript as follows:

Female mice were not used for the behavioural tests because male and female siblings were not separated at any time before the experiments. Therefore, using adult female mice (at age 50 days) was not appropriate due to possible concerns about the effect of sexual maturation and potential gestation. Sibling separation was avoided in this study as the primary aim was to examine the negative impact of maternal separation and to rule out the possible effects inflicted by other stress factors, such as the separation of siblings

Comment 3: Finally, there does not seem to be a coherent style in the graphs (significance bar overlays with bar graph, missing error bars, different fonts, etc.) and the manuscript needs to undergo some major revision before re-submission.

The replies: All graphs were revised and adjusted in a coherent style in the revised copy of the manuscript.

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Comments and
Suggestions for

Authors

The present study by Jarrar *et al.* investigated the behavioral effects of prolonged maternal separation (MS) on the elevated plus maze (EPM) and the marble burying (MB) test. The authors found that separated mice exhibited decreased levels of anxiety in the EPM and an increase in compulsive burying activity in the MB. Unfortunately, I advise to reconsider only after major revision for the following reasons:

(1) I do not understand how this study relates to JPM, a journal that aims at bringing all aspects of personalized medicine to one platform. The authors also do not discuss this.

(2) The authors state that '*the behavioral effects of maternal separation (MS), ..., in both the EPM and MB tests remain incompletely known*'.

I do not understand how this study helps to shed light on this topic. Apart from several studies that investigated the effect of 'regular' periods of MS on anxiety, there are also some studies which investigated the effect of a prolonged period of MS (mainly about 20-21 days) on the EPM and other behavioral tests (e.g., Troakes & Ingram, 2008; meta-analysis by Wang *et al.*, 2020, *Transl Psychiatry* for summary; ...). Moreover, in contrast to the current study, most of the other studies included additional analyses, such as cFos mRNA expression etc., to provide insights into the neuronal mechanisms.

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Best regards

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Comments and Suggestions for Authors All comments were addressed.

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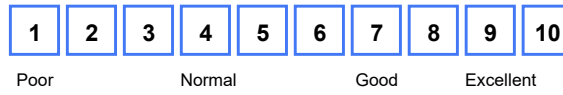


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Manuscript ID jpm-1922536

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Title Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice (https://www.mdpi.com/2075-4426/12/11/1921)

Authors Qais Jarrar * , Rami Ayoub , Kawther Alhussine , Khang Wen Goh , Said Moshawih , Chrismawan Ardianto * , Bey Hing Goh , Long Chiau Ming

Abstract Background: The elevated plus maze (EPM) and the marbles burying (MB) tests are common behavioral tests used for behavioral phenotyping of mouse models for neurodevelopmental disorders. However, the behavioral effects of maternal separation (MS), a standard paradigm for early life stress maternal separation; anxiety; compulsivity; elevated plus maze; marbles burying test; psychopa-ty; impulsivity tests. Methods: Male BALB/c mice were isolated from their mothers for 4 hours each day during the first 30 days after birth. On day 50 postnatal, mice were subjected to the EPM, and MB tests for behavioral evaluations. In addition, the locomotor activity of mice was evaluated using the actophotometer test. Results: The findings of the EPM test revealed that separated mice exhibited anxiolytic-like behaviors, as evidenced by a significant increase in the latency to closed arms and the time spent in the open arms compared with non-separated mice. Separated mice also showed compulsive burying activity in the MB test, as determined by a significant increase in the number of buried marbles. The results of the actophotometer test did not show any significant change in locomotor activity. Conclusions: Prolonged MS caused adult offspring of mice to exhibit a decrease in anxiety state and increased compulsive burying activity, which were not associated with a change in locomotor activity. Further investigations with validated tests are needed to support these findings.

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Authors' Responses to Reviewer's Comments (Reviewer 2)

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Author's Notes **Reviewer 2**

Comment 1: The introduction presented in the article does not describe properly the research question. Specifically, it lacks an appropriate description of the known effects of maternal separation on both humans and animals (see: McCall et al., 2019 ; Suomi et al., 1971).

The replies: The effects of maternal separation on both humans and animals were described in the introduction section of the revised manuscript as follows:

One potential of childhood negative experiences is the separation from the mother. It has been suggested that early separations impair the mother's ability to form an attachment, which reduces the possibility that the child and mother will develop a secure relationship (Network, 1997). The correlation between maternal separation and aberrant behaviors in humans was early recognized by John Bowl (1951) who suggested that continual disruption of the attachment between infants and mothers was associated with a high risk of cognitive impairment and affectionless psychopathy. Later studies found that children living in institutions with little chance for caregiver-child interaction grew more slowly in terms of their physical, cognitive, and social-emotional abilities and had a higher rate of behavioral and executive function issues, even years after adoption (Cramm et al., 2019; McCall et al., 2019). Epidemiological studies also showed that early MS is associated with a higher risk of developing schizotypal personality disorder symptoms (Anglin, Cohen and Chen, 2008), frequent nightmares (Csóka et al., 2011), and alcohol and drug dependence in early adulthood (Enoch, 2011). Moreover, there is evidence that MS caused differential impacts on fear-related behaviors in a sex and age-specific way (Toledo-Rodriguez and Sandi, 2007).

During the last decades, animal research has been extensively used to investigate CA effects, with a greater emphasis on the maternal separation (MS) paradigm (Lehmann et al., 1999). From an experimental viewpoint, MS is defined as a procedure employed to isolate newborns from their mothers for a predetermined period, which might range from a few hours to many days. A brief and short separation procedure entails a daily separation of 15 min over a period that can be extended to 14 days



(Arborelius and Eklund, 2007). In contrast, a severe and prolonged separation procedure entails a daily separation of 3–4 h, that lasts for more than 14 days (Sousa et al., 2014). Several studies have shown that MS consequence varies based on multiple variables, such as the time and duration of separation, as well as the animal species and strains used in different research groups (Romeo et al., 2003; Millstein and Holmes, 2007; Tractenberg et al., 2016; Tan et al., 2017; Miragaia et al., 2018). However, there is a significant body of evidence from studies in animals that indicates MS can disrupt the hypothalamus-pituitary-adrenal axis function (Feng et al., 2011), induce long-term oxidative stress in the brain (Uysal et al., 2008) and cause lasting changes in emotion-related behaviors as well as impair the growth of areas that involved in the stressful stimuli (Sullivan and Opendak, 2021). Neurochemical disorders (Hui et al., 2011), spatial memory loss (Grochecki et al., 2022), impulsivity (Colorado et al., 2006) and risk-taking behaviors such as drug abuse (Delavari et al., 2016) were also correlated to effect of MS.

Comment 2: The interpretation of the results regarding OCD is maybe just one possibility, but there are others such as Conduct disorder and attachment disorder. This should be discussed, and the OCD interpretation should tune down

The replies: We appreciate the reviewer for bringing up this issue. However, this study did not aim to examine the aggressive and anti-social behaviors which are fundamental components of conduct and attachment disorders. In light of this, we are unsure if the results of the current study can be utilized to demonstrate a direct relationship between MS and conduct- or attachment issues. On the otherhand, our study showed a positive correlation between prolonged maternal separation and excessive marble-burying activity which has been frequently interpreted by various research groups as an indicator of compulsive-like behavior in mice. As advised by the reviewer, the OCD interpretation was toned down in the revised manuscript.

Comment 3: The study is lacking any biological measures, that could have significantly improved the statement and validity of the results. For example, measurements of Cortisol levels could have strengthened the interpretation of the behavior as related to anxiety. Different measures from other or the same behavioral tests could have also added important information regarding the nature of the differences in behavior. Please consider adding some more parameters from the already conducted tests.

The replies: Many thanks to the reviewer for raising this concern. Unfortunately, I am afraid that it is not possible to perform the requested analyses at the present time as animals were dissected and no more plasma, serum and tissue sample are available. This part of analysis will be considered in the future work. In response to the reviewer's comment, we addressed this issue and other unavoidable limitations in the revised manuscript as follows:

Although this study has reached its aims, there were some unavoidable limitations. First, this research was restricted to exploring the alterations in behavioral phenotypes. Therefore, further studies are needed to involve some molecular evaluations. Second, our results did not determine whether the behavioral alterations in this study were attributed to the effect of MS itself or whether they were indirectly related to the stress inflicted on the mothers that could affect the care given to their pups. This could be solved by evaluating measures related to maternal care. Finally, as the male and female mice were not separated in the current study, the behavioral tests were conducted only on the male mice to avoid concerns related to the effect of sex maturation and possible gestation.

Comment 4:

-Methodology wasn't clear enough References to how maternal separation affects stress are missing. More references are needed.

The replies: More references were added and the maternal separation procedure was clarified as follows:

The procedure of MS was conducted as described previously (Cruz et al., 2008; Lundberg et al., 2017; Bian et al., 2021) with some modifications. Female pregnant mice were distributed individually into clean, transparent cages 3-6 days before giving birth. After birth, mice mothers were separated from their pups for 4 hours daily (between 9 a.m. and 1 p.m.) for 30 days or were left undisturbed (in the case of the control group), only handled during cage cleaning. During separation, mice mothers were placed in separate cages with clean bedding and free access to food pellets and drinking water. After day 30 postnatal, pups were kept in their cages and raised with their dams and siblings without any separation. All mice (dams and pups) were kept at room temperature (22-25 °C) and relative humidity (50-52%). Control



mice (non-separated offspring) were subjected to the same condition without exposing to the maternal separation procedure. At day 50 postnatal, adult male mice were submitted to a variety of behavioral tests, including the EPM, MB, and the actophotometer tests. The behavioral tests were repeated in three different sets of animals (n=6/each group/ each set). Data obtained from maternally separated mice were compared with their counterparts of non-separated mice

- It is also important to clarify the choice of those specific ages of the mice, as well as when all the mice were separated (experimental group AND CONTROL!!) in order to avoid reproduction with the mother or between siblings. In addition, please add a detailed explanation for using only males in the experiment.

The replies: the reason for choosing this age were discussed in the revised manuscript as follows:

Although most previous studies focused on the effects of maternal separation during infancy and the early weaning period (from day 1-21 postnatal), this study, however, was designed to examine the effect of a prolonged separation procedure that covered the prepubertal period (from day 1-30 postnatal). Research on brain development suggested that the maturing brain during prepubertal and adolescence period may be particularly vulnerable to the effect of stress, and that effect may significantly increase the risk of developing risky behaviors later in life (Winters and Arria, 2011) and cause differential impact on fear-related behaviors in a sex- and age-specific way (Toledo-Rodriguez and Sandi, 2007). Similarly, this study showed an evidence that prolonged MS reduced the anxiety level which reflects a risky-taking behavior.

-In addition, please add a detailed explanation for using only males in the experiment. Since the sex identification is challenging (between females and males) in early ages in mice, it is reasonable that until the age of approximately 20 days, the separation had to be conducted on both males and females (please clarify this in Methods). For that reason, it is not clear why female mice were excluded, as the information of differences or lack of it between the sexes could have been important.

The replies: the reason for using only male mice were explained in the revised manuscript as follows:

Female mice were not used for the behavioural tests because male and female siblings were not separated at any time before the experiments. Therefore, using adult female mice (at age 50 days) was not appropriate due to possible concerns about the effect of sexual maturation and potential gestation. Sibling separation was avoided in this study as the primary aim was to examine the negative impact of maternal separation and to rule out the possible effects inflicted by other stress factors, such as the separation of siblings.

-The Discussion Must include a much more detailed description of its limitations. Specifically, regarding maternal caregiving effect. It should be discussed whether the significant differences observed in the behavior of the separated mice were caused by the separation itself, and/or by the stress inflicted on the mothers which affecting their ability to care for the pups. This could have been solved by adding data and measures related to the maternal care.

The replies: the main limitations were added in the revised manuscript as follows:

Although this study has reached its aims, there were some unavoidable limitations. First, this research was restricted to exploring the alterations in behavioral phenotypes. Therefore, further studies are needed to involve some molecular evaluations. Second, our results did not determine whether the behavioral alterations in this study were attributed to the effect of MS itself or whether they were indirectly related to the stress inflicted on the mothers that could affect the care given to their pups. This could be solved by evaluating measures related to maternal care. Finally, as the male and female mice were not separated in the current study, the behavioral tests were conducted only on the male mice to avoid concerns related to the effect of sex maturation and possible

Comment 5: The most important statistics and data presentation: It is not clear why the results are presented as three trails separately. This is NOT acceptable. This indeed should be detailed described in the Methods but should be averaged in summarized graphs. Unless there is a difference between those trails, there is no reason to analyze them separately.

The replies: data and statistics are presented in the revised manuscript as indicated.

Minor comments



-In Figure 1 the position of * should be corrected.

The replies: corrected

-There is also an inconsistency in the titles of the graph's axis. In addition, the titles in the y-axis of Figures 3 and 4, should be changed from "amount of time..." to "time spent..."/ duration.

The replies: the titles of the graphs were changed in the revised manuscript and corrected as indicated.

-Please present a dot scatter in the graphs, which will provide further information regarding the variance within the group and the N sample size in each group.

The replies: done as indicated

Review Report Form

Quality of English () English very difficult to understand/incomprehensible
Language () Extensive editing of English language and style required
() Moderate English changes required
(x) English language and style are fine/minor spell check required
() I am not qualified to assess the quality of English in this paper

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	()	()	(x)	()
Are all the cited references relevant to the research?	()	(x)	()	()
Is the research design appropriate?	()	(x)	()	()
Are the methods adequately described?	()	()	(x)	()
Are the results clearly presented?	()	()	(x)	()
Are the conclusions supported by the results?	()	()	(x)	()

Comments and Suggestions for Authors
Jarrar et al. in their manuscript "Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice" are presenting significant behavioral differences in the elevated plus maze (EPM) and the marble burying (MB) tests between BALB/c male mice that have been separated from their mothers and a control group. In addition, they found that there is no significant locomotor difference between the groups in the actophotometer test.

The study found interesting results regarding the change in behavior caused by maternal separation. The lack of significant differences in locomotor activity is also important to support the conclusion regarding behavioral changes. Overall, this manuscript regards an interesting and important topic, and the study design seems logical. However, I do have some major and minor comments that have to be addressed.

Major comments

1. Introduction: The introduction presented in the article does not describe properly the research question. Specifically, it lacks an appropriate description of the known effects of maternal separation on both humans and animals (see: McCall et al., 2019 ; Suomi et al., 1971).
2. Conclusions and Discussion: The interpretation of the results regarding OCD is maybe just one possibility, but there are others such as Conduct disorder and attachment disorder. This should be discussed, and the OCD interpretation should tune down
3. Study design: The study is lacking any biological measures, that could have significantly improved the statement and validity of the results. For example, measurements of Cortisol levels could have strengthened the interpretation of the behavior as related to anxiety. Different measures from other or the same behavioral tests could have also added important information regarding the nature of the differences in behavior. Please consider adding some more parameters from the already conducted tests.



4. **Methods:** methodology wasn't clear enough. References to how maternal separation affects stress are missing. More references are needed. It is also important to clarify the choice of those specific ages of the mice, as well as when all the mice were separated (experimental group AND CONTROL!!) in order to avoid reproduction with the mother or between siblings. In addition, please add a detailed explanation for using only males in the experiment. Since the sex identification is challenging (between females and males) in early ages in mice, it is reasonable that until the age of approximately 20 days, the separation had to be conducted on both males and females (please clarify this in Methods). For that reason, it is not clear why female mice were excluded, as the information of differences or lack of it between the sexes could have been important. The Discussion Must include a much more detailed description of its limitations. Specifically, regarding maternal caregiving effect. It should be discussed whether the significant differences observed in the behavior of the separated mice were caused by the separation itself, and/or by the stress inflicted on the mothers which affecting their ability to care for the pups. This could have been solved by adding data and measures related to the maternal care.
5. **The most important statistics and data presentation:** It is not clear why the results are presented as three trails separately. This is NOT acceptable. This indeed should be detailed described in the Methods but should be averaged in summarized graphs. . Unless there is a difference between those trails, there is no reason to analyze them separately.

Minor comments

In **Figure 1** the position of * should be corrected. There is also an inconsistency in the titles of the graph's axis. In addition, the titles in the y-axis of **Figures 3 and 4**, should be changed from "amount of time..." to "time spent..."/ duration. Please present a dot scatter in the graphs, which will provide further information regarding the variance within the group and the N sample size in each group.

Submission Date 01 September 2022
Date of this review 13 Oct 2022 05:48:51

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Review Report rating refer to the guideline:

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	Journal	JPM (https://www.mdpi.com/journal/jpm) (ISSN 2075-4426)	Overview
	Manuscript ID	jpm-1922536	Contact Careers
	Type	Article	
	Title	Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice (https://www.mdpi.com/2075-4426/12/11/1921)	
	Authors	Qais Jarrar * , Rami Ayoub , Kawther Alhussine , Khang Wen Goh , Said Moshawih , Chrismawan Ardianto * , Bey Hing Goh , Long Chiau Ming	
	Abstract	Background: The elevated plus maze (EPM) and the marbles burying (MB) tests are common behavioral tests used for behavioral phenotyping of mouse models for neurodevelopmental disorders. However, the behavioral effects of maternal separation (MS), a standard paradigm for early life stress maternal separation; anxiety; compulsivity; elevated plus maze; marbles burying test; psychopa-ty; impulsivity tests. Methods: Male BALB/c mice were isolated from their mothers for 4 hours each day during the first 30 days after birth. On day 50 postnatal, mice were subjected to the EPM, and MB tests for behavioral evaluations. In addition, the locomotor activity of mice was evaluated using the actophotometer test. Results: The findings of the EPM test revealed that separated mice exhibited anxiolytic-like behaviors, as evidenced by a significant increase in the latency to closed arms and the time spent in the open arms compared with non-separated mice. Separated mice also showed compulsive burying activity in the MB test, as determined by a significant increase in the number of buried marbles. The results of the actophotometer test did not show any significant change in locomotor activity. Conclusions: Prolonged MS caused adult offspring of mice to exhibit a decrease in anxiety state and increased compulsive burying activity, which were not associated with a change in locomotor activity. Further investigations with validated tests are needed to support these findings.	

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Authors' Responses to Reviewer's Comments (Reviewer 2)

Author's Notes

Dear Reviewer,

"Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice" JPM-1922536

Thank you very much for your valuable suggestions and recommendations concerning the above manuscript. Herein is our reply to your comments:

Comment 1: In future studies biological measurements would improve the interpretation of the behavioral differences observed in the current experiment. We specifically suggest using cortisol measurements as additional information regarding changes in stress response due to maternal separation. Changes in cortisol function have been linked to maternal separation and early childhood stress in both animals and humans (see: de Weerth et al., 2013 ; Feng et al., 2011 ; Tops et al., 2007). Other biological measurements especially related to the function of the Amygdala and pre-frontal cortex should also be considered.

The answer: We included the suggested modification in the revised manuscript as kindly indicated by you.

Comment 2: We suggest that in future research, both behavioral and biological measurements would be considered to assess maternal care and stress. As this could contribute to the differences in the behavior of the offspring.

The answer: We greatly appreciate this valuable suggestion and it will considered in our future work. However, we included the suggestion in the revised manuscript.

Comment 3: In a future study we would suggest measuring differences in both females and males. Although it is true that in the female population hormonal changes could influence results, the information on potential differences, or lack of, between the sexes is important to understand the influence of early maternal separation in later developing psychopathology. In addition, as the separation of both females and males is required in the currently used method, it would be a waste not to measure both sexes that have already experienced the experimental method.



The answer: We include in the revised manuscript the importance of testing both sexes concerning the influence of early maternal separation in later developing psychopathology. This will be considered in our future work.

Comment 4: We suggest considering the theory of attachment (Bowlby, 1979) as a theoretical framework for this and future maternal separation experiments. Attachment theory has extensive empirical support regarding the human maternal-infant early relationship and its effects on later development.

The answer: The revised copy of the manuscript shows the interpretation of the current findings with the context of the attachment theory.

Best regards

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Quality of English () English very difficult to understand/incomprehensible
 Language () Extensive editing of English language and style required
 () Moderate English changes required
 (x) English language and style are fine/minor spell check required
 () I am not qualified to assess the quality of English in this paper

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	(x)	()	()	()
Are all the cited references relevant to the research?	()	(x)	()	()
Is the research design appropriate?	()	(x)	()	()
Are the methods adequately described?	()	(x)	()	()
Are the results clearly presented?	(x)	()	()	()
Are the conclusions supported by the results?	()	(x)	()	()

Comments and Suggestions for Authors
 Jarrar et al. manuscript "Prolonged Maternal Separation Reduces Anxiety State and Increases Compulsive Burying Activity in the Offspring of BALB/c Mice" indeed improved, but further discussion of future research plans will benefit the manuscript. We propose some minor changes in the Discussion.

Minor changes:

1. In future studies biological measurements would improve the interpretation of the behavioral differences observed in the current experiment. We specifically suggest using cortisol measurements as additional information regarding changes in stress response due to maternal separation. Changes in cortisol function have been linked to maternal separation and early childhood stress in both animals and humans (see: de Weerth et al., 2013 ; Feng et al., 2011 ; Tops et al., 2007). Other biological measurements especially related to the function of the Amygdala and pre-frontal cortex should also be considered.
2. We suggest that in future research, both behavioral and biological measurements would be considered to assess maternal care and stress. As this could contribute to the differences in the behavior of the offspring.
3. In a future study we would suggest measuring differences in both females and males. Although it is true that in the female population hormonal changes could influence results, the information on potential differences, or lack of, between the sexes is important to understand the influence of early maternal separation in later developing psychopathology. In addition, as the separation of both females and males is required in the currently used method, it would be a waste not to measure both sexes that have already experienced the experimental method.
4. We suggest considering the theory of attachment (Bowlby, 1979) as a theoretical framework for this and future maternal separation experiments. Attachment theory has extensive empirical support regarding the human maternal-infant early relationship and its effects on later development.



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Poor

Normal

Good

Excellent

