Research Article

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Journal of Translational Neurosciences
ISSN 2573-5349

2019
Vol.4 No.1:1

DOI: 10.21767/2573-5349.100023

A Novel Simple Method for Assessing Rodent Innate Fear Using Round Elevated Platform

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Abstract

Open field (OF) and elevated plus maze (EPM) have been widely used for assessing rodent innate fear. In OF task, animals with more fear will spend less time in the center zone (unsafe zone). Regarding EPM, animals with more fear will spent less time in the open arms (unsafe zone). Thus less activity in unsafe zone will be observed in animals with more fear. Based on this assumption, we speculated that our previously designed opaque round elevated platform (O-REP) with open space can be used for assessing rodent innate fear by analyzing animal activity. In O-REP, height and the open space make the animals feel fearful in O-REP and decrease its activity. To verify this novel method, the difference between female and male mice were examined by OF, EPM, and O-REP respectively. The results showed that EPM task could not find the significant fear difference between female and male mice using ICR and C57/BL6 strain. However, C57/BL6 female mice displayed less fear both in OF and O-REP task, which indicates that OF and O-REP task displayed more sensitivity than EPM task in assessing rodent innate fear. Furthermore, O-REP task rather than OF task found that female ICR mice displayed significantly more innate fear in O-REP, which indicated that O-REP might be more sensitive than OF and EPM in assessing innate fear. Further investigations showed that the animals displayed similar activities in O-REP, opaque square elevated platform (O-SEP) and transparent round elevated platform (T-REP), indicating that shape and transparency of EP did not affect the sensitivity of EP. Thus our data demonstrated that the REP is novel alternative task for evaluating rodent innate fear, which sensitivity was not affected by the shape and transparency. The present studies will greatly facilitate fear related research.

Keywords: Innate fear; Open field; Elevated plus maze; Round elevated platform

Received: November 20, 2018; **Accepted:** November 29, 2018; **Published:** December 06, 2018

Citation: Ding XF, Zhao YQ, Peng W, Li H, Shao NS, et al. (2019) A Novel Simple Method for Assessing Rodent Innate Fear Using Round Elevated Platform. J Transl Neurosci Vol.4 No.1:1

Introduction

Fear encompasses both learned fear and innate fear, which is normal emotion with great adaptive value that has been selected along the evolutionary process [1-3]. Though both learned and innate fear responses are controlled by the amygdala complex, they are definitely different. Learned (Acquired) fear triggers characteristic behaviors of escape and avoidance in response to a specific, previously experienced stimulus, such as pain or the threat of pain. In contrast, innate fear is genetically encoded and does not require response learning [2]. Our understanding of learned fear is largely based on studies of Pavlovian fear conditioning, in which an initially neutral conditioned stimulus (CS) of any sensory modality (such as sound) is paired with an innately aversive unconditioned stimulus (Such as electric foot shock) [4-7]. To test learned fear level, freezing time after CS was used as index for characterizing fear level [8-10]. Different from learned fear, entries into and time spent in the unsafe environment was usually to assess the animal innate fear. Based on this assumption, open field (OF), light-dark box (LDB), elevated

plus maze (EPM), Social interaction test was usually used to examine the rodent innate fear [1,11-14]. All of the above tasks can simultaneously provide a relative safe (familiar) area and an unsafe (unfamiliar) environment, which allow the animals to freely approach the novel area (open space, height and bright lit) to satisfy its curiosity, while avoid it when feeling afraid. Thus the psychological conflict between exploring drive and motive to avoid aversive unsafe environment was used to reflect animal anxiety, and the exploring time in unsafe zone was used to reflect innate fear [15-17]. The more time spent in the unsafe zone, the less fear level will be displayed by the animals. However, due to the different compartment settings, the results from these apparatus are sometimes inconsistent. In addition, for all mammalians, Security requirements was the first requirement should be satisfied. Thus, the safe areas of the existing apparatus will decrease the motive to explore in the unsafe zone, which make them not sensitive enough to examine animal innate fear difference in some content. For OF task, walls provide animals a safe area and animals spent most of test duration in this area. They will feel safe in this area and may be not willing to explore in the center zone, other than afraid to explore. In the EPM, close arms provide a dark and safe compartment, while the open arms provide an unsafe zone. According to its principle, the more time spent in the open arms, the less anxiety and fear will be displayed by the animals. However, rodent prefer staying in the dark environment, thus sometimes, animals doesn't travel to open arms may be due to their preference in dark zone, but not only due to its fear. In our experiments, some mice even stay in the closed arm during the all test duration and never enter into the open arms. For the light-dark box, it comprises a light box (aversive area) and a dark box. Similar with EPM, animal prefer staying in dark environment, thus less entries into or time spent in the light box doesn't necessarily mean that they feel fearful. In Social interaction test, familiar mouse may satisfy its social requirement and no need to approach the unfamiliar mouse [18-20]. Thus these limitations greatly affect their effectiveness in assessing animal fear. To overcome these limitations, we use an Opaque Round Elevated Platform (O-REP, 40cm above the floor) with open space to evaluate rodent innate fear, which has been used for examining rodent anxiety previously [21]. Because there is no walls and enclosed compartment was designed in this apparatus, so the mice on the O-REP will have no safe zone to hide and feel rather afraid, which will results in decrease of activity (travel less distance). Thus the travelling distance in O-REP may be used for reflecting rodent innate fear. The outer zone of O-REP is similar with the open arm of EPM, and the open space is similar with the center zone of OF apparatus, all of which were utilized to produce fearful challenge [22]. Thus the fearful challenge in O-REP should be higher than that in OF and EPM. As we know, dangerous environment is easier than safe environment to discriminate brave animals and less brave animals. Therefore, the O-REP may be more sensitive in finding innate fear difference between different groups. Due to the different fear level between female and male [23-25], to test our O-REP task, innate fear difference between female and male mice was examined by EPM, OF and O-REP, and the data from these three tasks was compared. In addition, the effect of shape and transparency of the EP was also investigated, in hope that we can provide researchers a novel sensitive method for assessing rodent innate fear.

Materials and Methods

Animals

C57/BL6 (10 mice female and 10 male mice) and ICR mice (10 female and 10 male mice) was originally purchased from Jackson laboratory and in bred in the Animal Research Facility of Kansas City Veterans Affairs Medical Center (KCVAMC). All animals were socially housed on a 12-hour light/dark cycle, with free access to rodent chow and water until 1 week prior to behavioral testing. One week before the tests, these animals were transferred to behavioral testing room for adaptation to the new environment. Behavioral testing was performed during the light period (8:00-12:00 AM). All the procedures for using these animals were approved by the Institutional Animal Care and Use Committees of KCVAMC.

Open field

Individual mouse was placed at the center of an open square chamber (40 cm×40 cm) with walls of 40 cm height (Med Associate INC, St. Albans, USA), and the total travel distance, the entries into the center zone, and the time spent in center zone of the animals was recorded for 5 minutes (min) each trail using a video camera (Fujinon, IL, USA) combined with ANY-maze™ Video Tracking System (Stoelting, Wood Dale, IL, USA) [26,27].

Elevated plus maze

The EPM (Med Associate INC, St. Albans, USA) consists of two open arms, two closed arms, and a center zone at the intersection of four arms elevated 40 cm above the ground. For testing, animal was placed in the center zone of the maze facing a closed arm and allowed to explore the maze for 5 min each trial. Animal activity was recorded by video tracking system combined with ANY-maze™. Time spent in open arms was analyzed to assessing the animal innate fear as previously described [21,28].

Elevated platform

As previously described, the round opaque EP (O-REP) was elevated 40 cm above the floor to produce similar strength of fearful challenge with that of EPM, and the radius was designed to be 22.5 cm (area= π x 22.5^2) to provide the almost same activity area with that of OF (40 cm x 40 cm) [21]. In addition, no walls were designed on the O-REP, and only 0.3 cm high edge was designed to preventing the mouse from slipping and falling off the O-REP. Thus the open space and height will make the animals feel afraid and subsequently decrease its activity. The total travel distance and immobile time was recorded to analyze the animal fear. Animals with less fear will travel more distance on the O-REP. Furthermore, to investigate whether the shape and the transparency affect the effect of the EP in assessing rodent fear, an opaque square elevated platform (O-SEP, 40 cm x 40 cm, opaque) and a transparent round elevated platform (T-REP, area= π x 22.5^2) was designed. Regarding the T-REP, the center area

(area= π x 7.5^2) was opaque and defined as a start zone, while the rest area was designed to be transparent to produce more fear. Similar with O-REP, there is also no walls were designed in O-SEP and T-REP. Rodent locomotor activity on these three kinds of EP was analyzed and compared.

Statistical analysis

The differences between any two animal groups were analyzed by two-tailed t-tests. Linear correlation analysis was also performed to analyze the correlation between OEP and SEP/TEP. The Graph Pad prism 5.0 was used for calculating data and Statistic analysis. All quantitative results were expressed as mean ± SEM and P<0.05 was considering being statistically significant.

Results

Elevated plus maze was unable to find the significant difference of innate fear between female and male mice

Elevated plus maze was widely used for examining rodent fear and anxiety. The EPM consist two open arms and two closed arms. Elevated open arms provide an unsafe but curious area for the rodent, while the closed arms provide rodent a dark and enclosed compartment to satisfy security requirement. Animals with less anxiety and fear level will spend more time in the open arms. To discuss the possibility of assessing rodent fear using EP, we first examined the fear difference between female and male mice using EPM. As shown in **Figure 1A and B**, no significant difference of entries into and time spent in the open arms was observed between C57/BL6 female mice and male mice. Additionally, there is no significant difference of entries into and time spent in the open arms was observed between ICR female mice and male mice (as shown in **Figure 1C and D**). The results demonstrated that EPM was not sensitive enough to find significant innate fear difference between female and male mice.

EP is more sensitive than OF in assessing innate fear difference between female and male mice

OF is another widely used task for evaluating rodent fear. The center zone of OF apparatus provides animals a novel unsafe environment to satisfy their curiosity, and entries into and time spent in the zone indicates the fear level of animals. Animals with less fear level will enter the center zone more frequently, travel more distance, and spend more time in this zone. Therefore, we next examined fear difference between female and male mice using OF task. As shown in **Figure 2A and B**, regarding C57/BL6 mice, female mice entered the center zone significant more frequently (P<0.0001) and spent much more time (P<0.05) than

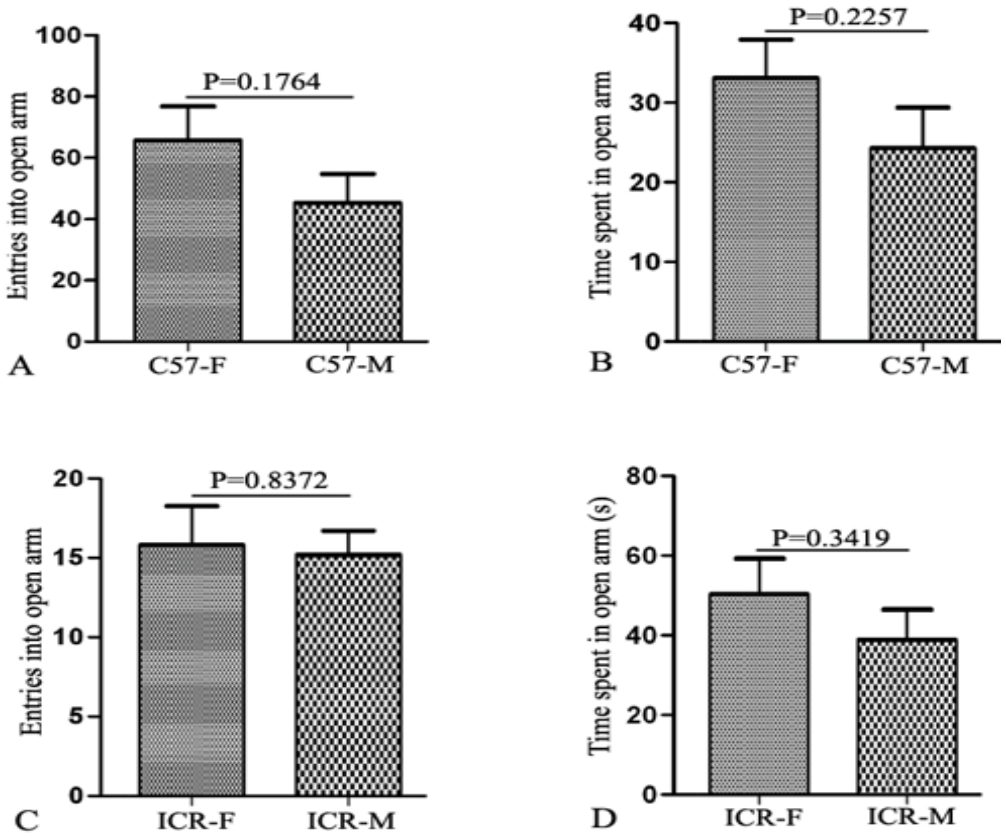

Figure 1 Examining the difference of innate fear between female and male mice with EPM task. A: No significant difference of entries into the open arms was found between C57/BL6 female and male mice. B: No significant difference of time spent in the open arms was found between C57/BL6 female and male mice. C: No significant difference of entries into the open arms was found between ICR female and male mice. D: No significant difference of time spent in the open arms was found between ICR female and male mice.

male mice. Further analysis showed that C57/BL6 female mice travelled remarkably more distance than male mice in OF (**Figure 2C**, P<0.01). Thus the results showed that female C57/BL6 mice displayed significant less fear than male mice in OF task. Next we examined the fear difference between C57/BL6 female and male mice with O-REP task. According to the principle of O-REP, animals with higher fear level will show less activity. Thus we analyzed animal fear level by analyzing total travel distance in O-REP task. As shown in **Figure 2C**, C57/BL6 female mice travelled remarkably more distance than male mice in O-REP task (P<0.01), which is consistent with the results in OF task, and thus indicating that O-REP may be feasible to be used in assessing animal fear level. Furthermore, we found that both female and male C57/BL6 mice greatly decreased their activity in O-REP task compared with that in OF task, which demonstrated that higher fearful challenge was produced in O-REP, which is consistent with the principle of O-REP. To further test O-REP task, another mouse strain (ICR mouse) was used, and the difference of fear between female and male mice was evaluated with OF and O-REP task respectively. As shown in **Figure 2D and E**, no significant difference of entries into (P=0.2051) and time spent (P=0.3715) in the center zone was found between ICR female and male mice in OF task. Further analysis showed that no significant difference of travelling distance between ICR female and male mice was observed in OF task (P=0.5330). However, using O-REP task, we found that ICR male mice travelled obviously more distance than female mice (**Figure 2F**, P<0.05), indicating that male ICR mice display less fear level than female ICR. Similarly, we found both male and female ICR mice decreased their moving distance in O-REP task compared with that of OF task (**Figure 2F**). Thus the results demonstrated more fearful challenge was produced in O-REP task than that of OF task, and using O-REP task for assessing rodent fear is feasible. Most importantly, O-REP shows higher sensitivity than OF in assessing animal innate fear.

The shape of elevated platform does not affect its sensitivity in assessing rodent innate fear

Though the O-REP was proved to be an effective apparatus for assessing rodent innate fear, the effect of the shape of O-REP was unknown. To this end, we designed an opaque square elevated platform (O-SEP) with open space, the area of which is equal to the area of the round EP (O-REP) and OF (40 cm x 40 cm, **Figure 3A**). To compare O-SEP with O-REP, the locomotor activity of 10 ICR male mice was tested with OF, O-SEP and O-REP respectively. As shown in **Figure 3B**, the total travelling distance was greatly decreased in O-REP and O-SEP compared with that in OF, which indicated that the more fearful challenge was produced by the O-SEP and O-REP, which is consistent with the principle of EP. Further analysis showed that no significant difference of moving distance was found between O-REP and O-SEP, indicating the similar locomotor activity of animals in O-SEP and O-REP. to further compared O-SEP and O-REP, both of the O-SEP and O-REP were divided into three consecutive zones using Any-maze tracking system according our previously work [21], including center, middle and outer zone, and we compared the difference of the time spent in each zone between O-SEP and O-REP. As

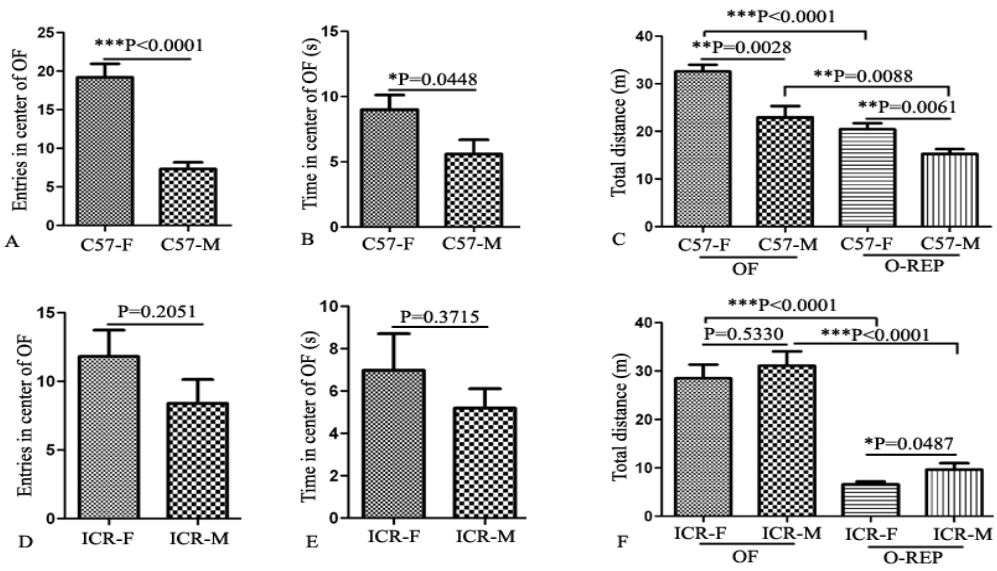

Figure 2 Examining the difference of innate fear between female and male mice with OF task and EP task. A: C57/BL6 female mice entered into the center zone of OF more frequently compared with male mice (P<0.0001). B: C57/BL6 female mice spent remarkably more time the center zone of OF compared with male mice (P<0.05). C: C57/BL6 female mice travelled more distance in OF and O-REP compared with male mice (P<0.01). Both C57/BL6 female (P<0.001) and male (P<0.01) mice travelled significant less distance in O-REP compared with that in OF. D: No significant difference of entries into the center zone of OF was found between ICR female and male mice. E: No significant difference of time spent in the center zone of OF was found between ICR female and male mice. F: No significant difference of total moving distance between ICR female and male mice was found in OF, but O-REP task did find that ICR female mice travelled less distance in EP compared with male mice (P<0.05). Likewise, we found both ICR female and male mice travelled significantly less distance in EP than that in OF (P<0.0001).

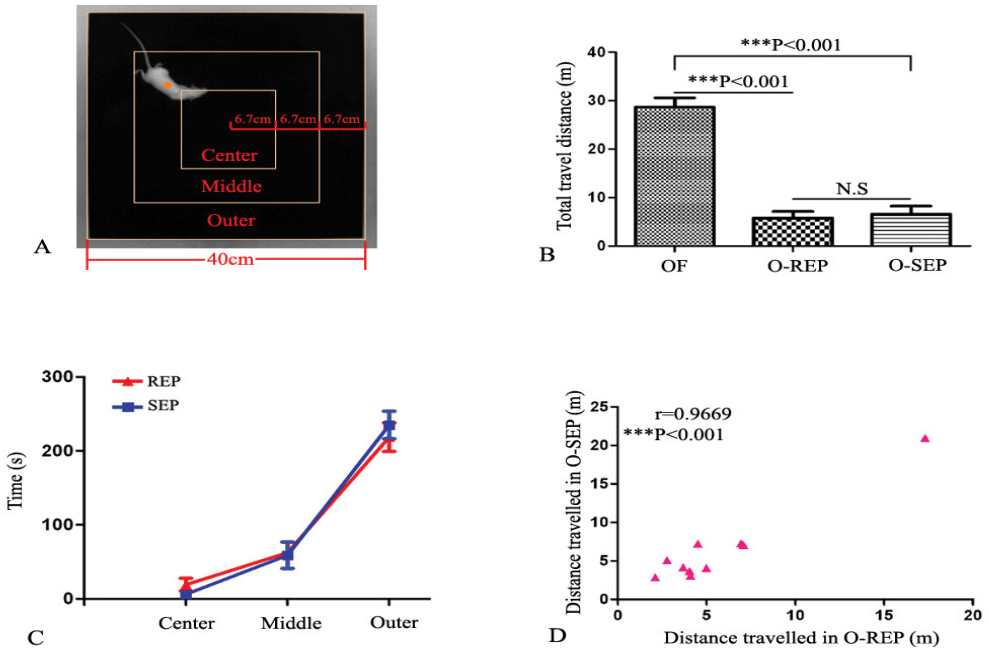

Figure 3 Comparison of O-SEP and O-REP in assessing animal innate fear. A: the overview and parameters of O-SEP, the O-SEP was divided into three consecutive zones and elevated 40 cm above the floor. B: Distance travelled in O-SEP and O-REP is significantly less than that in OF, but no significant difference of distance was found between O-SEP and O-REP. C: No significant difference of time spent in each zone was found between O-SEP and O-REP. D: Distance travelled in O-SEP was highly correlated with that in O-REP.

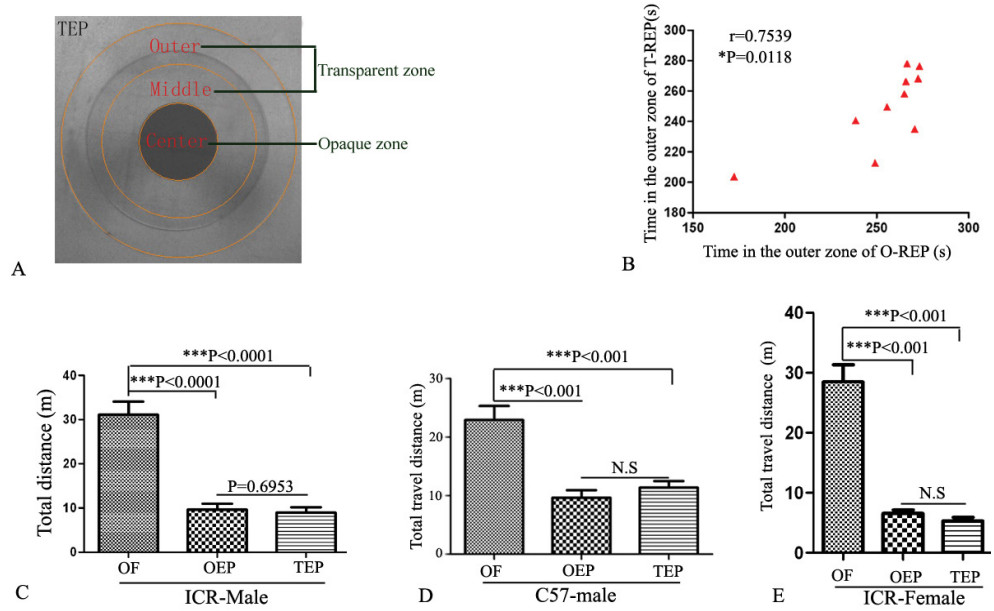

Figure 4 Comparison of T-REP (TEP) and O-REP (OEP) in assessing animal innate fear. A: the overview and parameters of T-REP, the T-REP was divided into three consecutive zones and elevated 40 cm above the floor. The center zone was opaque while the middle zone and outer zone was transparent. B: Time spent in the outer zone of T-REP was highly correlated with that in the outer zone of O-REP. C: Distance travelled in T-REP (TEP) and O-REP (OEP) is significantly less than that in OF, but no significant difference of distance using ICR-Male mice was found between T-REP (TEP) and O-REP (OEP). D: Distance travelled in T-REP (TEP) and O-REP (OEP) is significantly less than that in OF, but no significant difference of distance using C57-Male mice was found between T-REP (TEP) and O-REP (OEP). E: Distance travelled in T-REP (TEP) and O-REP (OEP) is significantly less than that in OF, but no significant difference of distance using ICR-Female mice was found between T-REP (TEP) and O-REP (OEP).

shown in **Figure 3C**, no significant difference of time spent in each zone was found between O-SEP and O-REP; most of time was spent in the outer zone of O-SEP and O-REP, indicating the similar moving path in these two kinds of EP. Correlation analysis showed that distance travelled in the O-SEP is highly correlated with that in O-REP (**Figure 3D**), indicating the similar locomotor activity of animal in O-SEP and O-REP. The plot of moving path in OF, O-SEP and O-REP also support the above results in **Figure S1**. Thus the results demonstrated that SEP is equal to REP in assessing rodent animal innate fear.

Transparency does not affect the animal activity on the EP

Previous work in other labs showed that mouse visual system could discriminate the transparent and opaque ground. According to the previously reported work, we speculated that, with same traveling area, transparent round elevated platform (T-REP) should produce more fearful challenge than opaque round elevated platform (O-REP), and thus the transparency should affect the sensitivity of EP in theory. To prove our speculation, we designed a transparent round elevated platform (T-REP) share the same area and same shape with O-REP, and tested the activity of 10 ICR male mice using OF, O-REP and T-REP respectively. As a result, we found that no significant difference of travelling distance was found between T-REP and O-REP, but the travelling distance was decreased in both T-REP and O-REP compared with that in OF, indicating that similar fearful challenge was produced by T-REP and O-REP. Additionally, to compare the difference between T-REP and O-REP, the O-REP and T-REP were divided into three consecutive zones using Any-maze tracking software, including center, middle and outer zone. As shown in **Figure 4A and B**, correlation analysis showed that time spent in the center zone of O-REP was significantly correlated with that of T-REP. Further analysis showed that ICR male mice displayed similar spontaneous activity in O-REP and T-REP task **(Figure 4C)**. Moreover, we compared the activities of other two mouse strain in OF, T-REP and O-REP, and the results also showed that no activity difference was found between T-REP and O-REP **(Figure 4D and E)**. Thus the results demonstrated transparency did not affect the sensitivity of EP in assessing rodent innate fear.

Discussion

Fear encompasses innate fear and learned fear, both of which play pivotal role in surviving animals. Learned fear triggers characteristic behaviors of escape and avoidance in response to previously experienced stimulus. In contrast, innate fear triggers the escape behavior in response to the unsafe environment or the predators. Thus the methods for assessing these two kinds of fear are different. Present methods for examining learned fear were based on pavlovian fear conditioning, and lots of the detailed protocol of which has been reported [8-10]. Methods for assessing innate fear was usually investigated by examining the animal response to potential threat or fearful environment, such as the height, the open space, bright lit, looming shadows, smell of predators, auditory threat cues[11-14]. OF and EPM task were based on this and have been widely used for assessing rodent innate fear. OF task provide an open space in the center, and rodent feel afraid in this area, thus the time spent in this zone can reflect the rodent innate fear. Animals with less innate fear will spend more time in the center zone of OF. Regarding EPM, open arms provide the height to make the animal feel afraid, and animals with less innate fear will spent more time in the open arms. However, these two methods are not sensitive enough and the results from the two methods are sometimes inconsistent, which limit the innate fear research. Previously, we designed a novel elevated platform (O-REP) for assessing rodent anxiety and locomotor activity [21]. Recently, we found that mouse travelled remarkably less distance in this O-REP task than that in OF task, which indicated that more fearful challenge was produced in O-REP compared with that in OF, thus urge us to discuss the feasibility of examining the rodent innate fear and anxiety by analyzing the intensity of animal activity in O-REP, and the animals with higher innate fear level will be afraid to move, thus should travel less distance. To test our speculation, the innate fear difference between female and male mice was examined by EPM, OF and O-REP task respectively. As shown in **Figure 1**, EPM was unable to find the fear difference between female and male mice, including C57/BL6 and ICR mice. However, OF task did find that C57/BL6 female mice displayed less innate fear than male mice, which is inconsistent with the results from EPM task. Excitedly we found that O-REP task also found the significant difference between C57/BL6 female and male mice, which indicated that O-REP task might be feasible to assess rodent innate fear. In contrast, OF task was unable to find the fear difference between ICR female and male mice, but O-REP did find significant difference of fear between them, indicating the higher sensitivity of O-REP in assessing innate fear. The greatly decreased moving distance of animals in O-REP indicated the higher fearful challenge produced in O-REP, which is consistent with our speculation. In addition, to investigate whether the shape affects the sensitivity of O-REP, an opaque square elevated platform (O-SEP,) was designed. The moving area of SEP was designed same as that of opaque round elevated platform (O-REP) and open field chamber. The travelling distance of animals in these two different elevated platform tasks was compared. As a result, we found that no significant difference of innate fear was found between O-SEP and O-REP. Moreover, though the moving path is different, trends of time spent in each zone of both SEP and REP are consistent. Therefore, the shape of EP did not affect the sensitivity in assessing rodent innate fear. In addition, previous study showed that mice were afraid to move on the transparent elevated floor of open field, indicating the transparent moving area may increase the fearful challenge of EP [29]. Thus we designed a transparent round elevated platform (T-REP), but no difference of travelling distance of mice was found between O-REP and T-REP (both are round, radius=22.5 cm), indicating the transparency does not affect the sensitivity of EP in assessing rodent innate fear, which is contradictory to the reported work [29]. Nevertheless, studies about elevated plus maze demonstrated that the rodent sight is horizontal and transparency did not affect animal activity, which support our present studies [30]. In addition, estrogen is a very potential factor that may affect the female behavior [31]. However, in the present study, behavioral tests in open field, elevated plus maze, and the elevated platform were completed in a day, and we mainly focus the behavioral difference between the different

behavioral tasks. Thus the phase of the estrous cycle may have little effect on the conclusion.

In summary, we developed a novel simple method with higher sensitivity for assessing rodent innate fear using REP. The shape and transparency does not affect the sensitivity of REP. This method will facilitate to quickly and effectively examine the rodent innate fear.

Acknowledgement

This work was supported by grants to W.Z.S. from the Medical Research and Development Service, Department of Veterans Affairs (1I01BX001067-01A2), the Alzheimer's Association (NPSPAD-11-202149);

Author Contributions

Ding XF, William Z. Suo and Ming Fan designed the experiment. Ding XF and Wei Peng performed the experiment. Yong-Qi Zhao and Ding XF prepared the manuscript. Hui Li and Ning Sheng Shao provided constructive suggestions in revising the manuscript.

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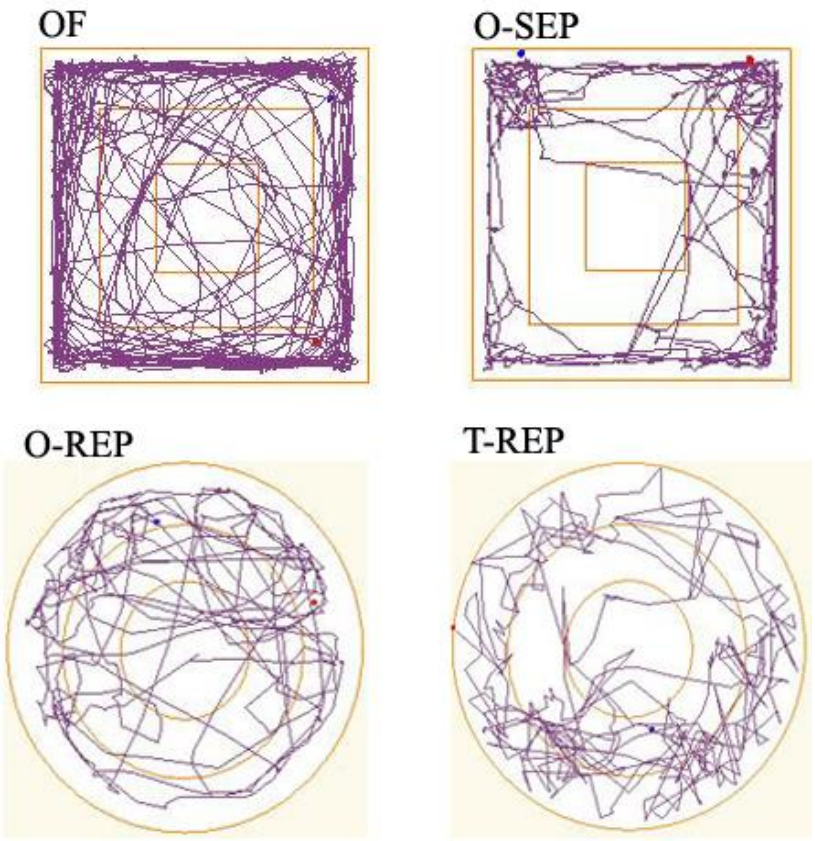
Figure S1



Supplementary Figure 1 The represent plot of moving path in OF, O-SEP, O-REP and T-REP task respectively.