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Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice

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HIGHLIGHTS

- The marble burying test is an effective assay to study the anxiogenic effect of stress.
- Increase in anxiety caused by 2-h acute stress was detected even 10 days later.
- This test may be used to study the gradual behavioral impact of stress over time.

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ABSTRACT

A majority of rodent studies characterizing the anxiogenic effects of stress have utilized exploration-based models, such as the elevated plus-maze. An alternative strategy has relied on ethologically natural behavior such as defensive burying. One such paradigm, marble burying, has proven to be an effective behavioral assay of the anxiolytic effects of pharmacological manipulations, and of genetically modified mouse models. Relatively little, however, is known about the sensitivity of this test in assessing the anxiogenic effects of stress. Most of the earlier reports have examined the immediate, but not more long-term, effects of pharmacological or environmental manipulations in mice. Hence, we used the marble burying test to examine if acute immobilization stress leads to enhanced anxiety-like behavior in C57Bl/6 mice if the test is employed with a significant time delay. We find this test to be sensitive enough to detect the anxiogenic effects even 10 days after a single episode of 2-h immobilization stress. Our results suggest that the marble burying test could serve as a useful behavioral paradigm for not only estimating the gradual progression of the anxiogenic impact of stress over time, but also raises the possibility of using the temporal delay after stress to test the potential efficacy of post-stress interventions with anxiolytic drugs.

1. Introduction

Debilitating and persistent emotional symptoms are a hallmark of stress-induced psychiatric disorders (DSM-IV, 2000). Animal models of fear, anxiety and depression have been used extensively to capture various aspects of these behavioral problems in rodents. Accumulating evidence shows that exposure to stress elicits anxiogenic effects in both rats and mice. The most widely used and well-established of these paradigms utilise alterations in exploratory behaviour as a read out of anxiety, such as the elevated plus-maze, wherein rodents are faced with a conflict situation between exploring open elevated arms and a natural tendency to hide in the enclosed arm (Mitra et al., 2005). Exposure to repeated stress results in animals spending less time and making fewer entries in the open arm compared to unstressed controls. Similar findings have been reported using the open-field and light–dark tests which also rely on a similar rationale (Kalueff et al., 2007; Goswami et al., 2013). Further, these animal models have been useful in testing the efficacy of anxiolytic agents. Indeed, these behavioral tests derive an aspect of their predictive validity from their responsiveness to anxiolytic drugs (Kalueff et al., 2007).

As useful as these behavioral assays have been in examining the neural basis of anxiety, they rely primarily on observations of exploratory behavior, and may not fully capture other facets of ethologically natural behavior in rodents. Defensive burying is part of a repertoire of defensive reactions that are naturally displayed by rodents (De Boer and Koolhaas, 2003). Burying of harmless objects such as glass marbles taps into this natural repertoire of rodent

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behaviour (Njung'e and Handley, 1991b). Importantly, the marble burying test has proven highly effective in detecting the efficacy of well-established anxiolytic agents ranging from benzodiazepines to selective serotonin reuptake inhibitors (Njung'e and Handley, 1991a). This test has also been used successfully to characterize anxiety-like behavior in genetically engineered mouse models (Gavioli et al., 2007). However, little is known about how environmental or behavioral perturbations that are known to enhance anxiety, such as exposure to stress, affect marble burying behavior. Moreover, earlier studies that investigated the effects of stress have used models of chronic stress (Dagytė et al., 2011). In addition, only the short-term effects, soon after the end of the stress or drug treatment, were measured in most studies. Hence, in the present study, we used a model of acute immobilization stress that has previously been reported to cause a delayed increase in anxiety in rats on the elevated plus-maze and open-field (Mitra et al., 2005). Our goal was to assess if the marble burying test would be sufficiently sensitive to detect an increase in anxiety-like behaviour in mice even 10 days after a single 2-h session of immobilization stress.

2. Materials and methods

2.1. Animals

Adult (45–60 days old) male C57Bl/6 mice (National Centre for Biological Sciences, Bangalore, India) were housed in groups of 2 or 3 in a standard cages 14 h light and 10 h dark schedule (lights on at 7:00 a.m.) with ad libitum access to food and water. All animal care and experimentation procedures were approved by the Institutional Animal Ethics Committee, National Centre for Biological Sciences (Approval No: SC-5/2009) and Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (Registration No: 109/CPCSEA).

C57Bl/6 mice were used in the present study as they have been used extensively in the marble burying paradigm in earlier reports (Homma and Yamada, 2009; Thomas et al., 2009). It forms an excellent background for genetic manipulations which have also been tested reliably using the marble burying paradigm (Zang et al., 2009; Sawin et al., 2014). For our purposes, which is a modulation of anxiety, the strain is useful being the most responsive to the anxiolytic diazepam in light–dark explorations and mounting defensive responses such as freezing and burying when challenged, without being classified as a 'highly-anxious' strain (Crawley et al., 1997; Yang et al., 2004). In strain comparisons of defensive burying, C57Bl/6 mice perform better at discriminative burying of the prod than most other strains tested (Harder and Maggio, 1983). More importantly, previous studies using immobilisation stress have also used this strain (Dong et al., 2004; Hill et al., 2013). Together, these earlier reports provide a solid foundation that we wanted to rely upon for the present study.

2.2. Stress protocol and experimental design

Mice were handled for the duration of weighing for 2 days prior to exposure to stress. Cages of mice were then randomly assigned to two experimental groups – control or stress such that all animals in a cage were subject to the same treatment. Mice in the stress group were subjected to acute immobilization stress, which consisted of a single session of complete immobilization for 2 h (between 10 a.m. and noon) in rodent immobilization bags without access to either food or water. Subsequently these stressed mice were allowed to recover stress-free for 10 days (Fig. 1A). Neither group of mice were handled during this period. On the 10th day after acute stress, both the stressed and unstressed control mice were subjected to the marble burying test (Day 10, Fig. 1A). All animals were weighed on the day of stress (Day 0, Fig. 1A), prior to the stress episode and again before the marble burying test 10 days later (Day 10, Fig. 1A).

Whole cages were assigned to either stress of control conditions. Thus, stressed and control animals were never caged together. Mice were stressed in a separate room and allowed to recover there for 1 h after the stress episode before returning to their housing room. Control animals were not present in this room during the administration of stress and were kept in their separate housing room. Further, the control and stress groups were maintained separately in individually ventilated cages (IVCs), thereby preventing common means of communication. Moreover, the separate IVCs containing the stressed and control groups were well-separated within the room with intervening barrier racks. All these measures helped in preventing ultrasonic and pheromonal communication between the two groups.

2.3. Marble burying test and data analysis

A cage (17.5 × 10 × 5.5 inches, Fig. 1B) was filled approximately 5 cm deep with husk bedding material that was evenly distributed into a flat surface across the whole cage. During the habituation phase of the test, the mice were introduced to the cage without any marbles and were allowed to explore it for 20 min and then removed. Twenty glass marbles (1.4 cm in diameter, plain dark glass) were then spaced evenly in a 4 × 5 grid on the surface of the bedding (Fig. 1B). During the testing phase each mouse was placed in the cage and allowed to explore it for 20 min. At the end of the test, mice were removed from the cage and the number of marbles buried with bedding up to 2/3 of their depth was counted. The entire test session was also recorded on video for later analysis offline. These recordings were scored for additional behaviours such as grooming, rearing, and digging. All analyses were performed blind.

2.4. Body weights

The net change in weights of mice between the beginning and end points of the experiments is presented as a percentage of initial weight.

2.5. Statistics

Burying over multiple time-points during the 20 min test was analyzed using two-way repeated measures ANOVA with post hoc Bonferroni's adjustment. For all other comparisons a paired Student's *T*-test was used. Values are expressed as mean ± SEM.

3. Results

Ten days after the 2-h acute immobilization stress (Fig. 1A), control and stressed mice were tested in the marble burying apparatus (Fig. 1B). At the end of the 20-min test period, the total number of marbles buried by the stressed mice was significantly higher than that buried by the control mice (Stress: 10.5 ± 1.2, $N = 8$; Control: 4.4 ± 1, $N = 9$, $p < 0.05$) (Fig. 2A). Binning the 20 min into 5 min intervals revealed that the stressed animals bury significantly more marbles in the first five minutes (Stress: 3 ± 0.7, $N = 8$; Control: 0.6 ± 0.2, $N = 9$; $p < 0.05$) (Fig. 2B). Moreover, this was a trend maintained over the course of the test such that the number marbles buried by stressed mice were higher at every time interval tested (Fig. 2B).

Consistent with this temporal profile of sustained enhancement in marble burying, the total amount of time spent in digging activity was also significantly higher in stressed animals (Stress: 71.2 ± 5.3 s, $N = 8$; Control: 34 ± 7 s, $N = 9$, $p < 0.01$) (Fig. 2C). Further, the latency to the first instance of digging the bedding material after being placed in the apparatus was shorter for stressed mice