

Video Article

Marble Burying and Nestlet Shredding as Tests of Repetitive, Compulsive-like Behaviors in Mice

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Abstract

Obsessive-compulsive disorder (OCD) and autism spectrum disorders (ASD) are serious and debilitating psychiatric conditions and each constitutes a significant public health concern, particularly in children. Both of these conditions are highlighted by the repeated expression of meaningless behaviors. Individuals with OCD often show checking, frequent hand washing, and counting. Children with ASDs also engage in repetitive tapping, arm or hand flapping, and rocking. These behaviors can vary widely in intensity and frequency of expression. More intense forms of repetitive behaviors can even result in injury (e.g. excessive grooming, hand washing, and self-stimulation). These behaviors are therefore very disruptive and make normal social discourse difficult. Treatment options for repetitive behaviors in OCD and ASDs are somewhat limited and there is great interest in developing more effective therapies for each condition. Numerous animal models for evaluating compulsive-like behaviors have been developed over the past three decades. Perhaps the animal models with the greatest validity and ease of use are the marble burying test and the nestlet shredding test. Both tests take advantage of the fact that the target behaviors occur spontaneously in mice. In the marble burying test, 20 marbles are arrayed on the surface of clean bedding. The number of marbles buried in a 30 min session is scored by investigators blind to the treatment or status of the subjects. In the nestlet shredding test, a nestlet comprised of pulped cotton fiber is preweighed and placed on top of cage bedding and the amount of the nestlet remaining intact after a 30 min test session is determined. Presently, we describe protocols for and show movie documentation of marble burying and nestlet shredding. Both tests are easily and accurately scored and each is sensitive to small changes in the expression of compulsive-like behaviors that result from genetic manipulations, disease, or head injury.

Video Link

The video component of this article can be found at <https://www.jove.com/video/50978/>

Introduction

Mouse models of human behaviors, and particularly psychiatric disorders, constitute important and essential experimental approaches to the study of disease mechanisms and to the development of new therapies. Many psychiatric illnesses remain extremely difficult to model in animals (e.g. schizophrenia). However, animals exhibit a large number of natural and distinctive behaviors that can readily be linked to similar behaviors in humans. Movement or locomotor activity is one example of a behavior in animals that has a counterpart in humans. Rodents often engage in a variety of repetitive behaviors and examples include grooming or digging. One psychiatric condition that involves the exhibition of recurrent and unwanted behaviors in humans is obsessive-compulsive disorder (OCD). Behaviors that are highly characteristic of OCD include checking, counting, and excessive washing or grooming. OCD is a highly prevalent (affects 1-3% of the population) and debilitating psychiatric disorder¹. A large number of pharmacological and behavioral approaches have been used to treat OCD. Many of these fall short of providing long-term relief and relapse is common at the end of a treatment regimen^{2,3}. Another psychiatric condition that involves the expression repetitive behaviors is autism spectrum disorder (ASD). ASDs are characterized by deficits in communication and social interaction and repetitive, stereotyped behaviors to include spinning, rocking, finger flicking or flapping, and complex body movements. The Centers for Disease Control and Prevention has estimated that about 1 in 88 children are diagnosed with ASDs. Treatment options for this disorder are extremely limited in number and efficacy^{4,5}. The intensity and frequency of occurrence of repetitive behaviors in OCD and ASD can become great enough to displace most other normal behaviors and social interactions in patients with these conditions. In light of the prevalence and debilitating nature of psychiatric conditions characterized by repetitive behaviors, and considering the poor understanding of their causes and limited treatment options, the use of animal models to study these behaviors takes on added urgency.

A large number of animal models of repetitive behaviors as seen in OCD and ASDs have been developed over the past 30 years and numerous excellent reviews describing these models have been published⁶⁻¹⁴. In general, these established models can be classified as genetic, pharmacological, neurodevelopmental and behavioral and most show good face, construct and predictive validity^{6,7}. We have used two behavioral models- marble burying and nestlet shredding- to assess repetitive behaviors in mice genetically depleted of serotonin^{15,16}. These methods have numerous advantages not the least of which are their ease of use, the accuracy with which the behaviors can be scored, the

spontaneous exhibition of these behaviors in rodents, and their use in high-throughput screening of genetically modified mice for abnormal behavioral phenotypes. Furthermore, each test is carried out in standard and familiar rodent housing cages containing the same bedding used in subjects' home cages, minimizing the need for habituation of mice to novel testing chambers. Food and/or water restriction are not required to "motivate" mice to perform. The marble burying test takes advantage of the proclivity of mice to dig in natural settings (e.g. burrows, escape tunnels) and in standard cage bedding, and the nestlet shred test capitalizes on the fact that mice are nesting animals by nature that build nests for protection of themselves and their offspring against environmental conditions (e.g. sound, light, temperature). Using nonspecialized and commonly available rodent housing materials, the marble burying and nestlet shredding tasks are simple and powerful behavioral assays of repetitive, compulsive-like behaviors in mice.

Protocol

All protocols using animals were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Care and Use Committee of Wayne State University (Permit Number A3310-01).

1. Preparation of Cages for Marble Burying and Nestlet Shredding

1. Use standard polycarbonate rat cages (26 cm x 48 cm x 20 cm) with fitted filter-top covers for the marble burying test. Add fresh, unscented mouse bedding material to each cage to a depth of 5 cm and level bedding surface by inserting another cage of the same size onto the surface of the bedding. This has the added advantage of impressing a template of parallel lines on the bedding surface that can be used for marble placement.
2. Place standard glass toy marbles (assorted styles and colors, 15 mm diameter, 5.2 g in weight) gently on the surface of the bedding in 5 rows of 4 marbles (see **Figure 1A**). Wash marbles in mild laboratory detergent, rinse exhaustively in distilled-deionized water, and dry prior to each use.
3. Use standard polycarbonate rat cages (19 cm x 29 cm x 13 cm) with fitted filter-top covers for the nestlet shredding test. Add fresh, unscented mouse bedding material to each cage to a depth of 0.5 cm and pack bedding by insertion of another cage of the same size onto the surface of the bedding.
4. Weigh commercially available cotton fiber nestlets (5 cm x 5 cm, 5 mm thick, ~2.5 g each) on an analytical balance and place one nestlet is placed on top of the bedding in each test cage (see **Figure 3A**).
5. Set stop watches or timers for timing session lengths (30 min for each test).

2. Animal Handling Prior to Testing

1. House mice individually or in groups in a room with a 12 hr light/dark cycle. Provide all mice with food and water *ad libitum*. Handle mice gently for three to five days prior to testing. Use mice of either sex for both tests.
2. Perform behavioral testing between 9:00 am and 4:00 pm and transport all home cages containing mice to the testing room 60 min before the start of testing.

3. Marble Burying Test

1. Place one mouse into a corner of the cage containing marbles, being careful to place the mouse on bedding as far from marbles as possible, and place the filter-top cover on the cage. Withhold food and water during the test. Allow mouse to remain in the cage undisturbed for 30 min.
2. Remove the mouse and return it to its home cage after test completion, taking extreme care not to move or dislodge the marbles in the process of removing the subject from the cage.
3. Task scorers (2-3) blind to the treatment conditions or genotype of the mouse being tested to count the number of marbles buried. Score a marble as buried if two-thirds of its surface area is covered by bedding. Average scores for the number of marbles buried for each mouse.
4. Retrieve all 20 marbles from the bedding. Dispose of bedding.

4. Nestlet Shredding Test

1. Place one mouse into a cage containing a single, preweighed nestlet and place the filter-top cover on the cage. Withhold food and water during the test. Leave mouse undisturbed in the cage with the nestlet for 30 min.
2. Remove mouse and return it to its home cage after test completion. Remove the remaining intact nestlet material from the cage with forceps and allow to dry O/N.
3. Weigh remaining unshredded nestlet and divide this weight by the starting weight to calculate percentage of nestlet shredded. If desired, score the form of the nest produced by the subject mouse using a definitive 5-point nest rating scale¹⁷ but realizing that this scale is generally intended for scoring nest building, not obsessive-like shredding.
4. Discard remaining shredded nestlet material and bedding.

Representative Results

A primary advantage of the marble burying and nestlet shredding tests is the fact that each depends on natural and spontaneous behaviors of mice that can be quantified after test completion, allowing a single investigator to set up an experiment and then remain outside of the testing room until sessions end. A critical component of the marble burying test is the careful removal of the subject from the cage after testing. Mice typically attempt to avoid capture and, in the process, can dislodge or move marbles that were partially buried or partially bury marbles that were not buried at the end of the test session. **Figure 1A** shows a test cage just prior to introduction of a subject, as viewed from above. We prefer a

precise alignment of marbles in this fashion which can subsequently be used to track movement of the marbles, in addition to burying (if desired). In an illustrative experiment, we compared marble burying by wild type (WT) mice to mice lacking the gene for tryptophan hydroxylase 2 (TPH2), the initial and rate limiting enzyme in the biosynthesis of brain serotonin (5HT). As a result of the null mutation in the gene for TPH2, these mice have no measureable brain 5HT^{15,16,18}. These mice show a very interesting behavioral phenotype characterized primarily by repetitive, compulsive-like digging and burying^{15,16}. **Figure 1B** shows a representative cage from WT mice and **Figure 1C** is a cage from a TPH2 knockout (KO) mouse after burying. The cage of the WT mouse is typical and shows few marbles buried (N = 3 in this example) and little displacement from their original locations. On the other hand, the TPH2 KO cage shows significant numbers of buried marbles (N = 17 in this example; unburied marbles marked with arrows) and an extensive displacement of marbles from their initial locations. The appearance of the bedding surface is also indicative of burying and digging behavior. **Figure 1D**, as viewed along the surface of the bedding, shows a level bedding surface prior to testing. **Figure 1E** shows that bedding also remains relatively undisturbed by a WT mouse. In contrast, **Figure 1F** shows a typical cage from a TPH2 KO mouse after the test session with a very undulating surface and deep troughs and peaks throughout the cage. **Figure 2** (reprinted from Angoa-Pérez *et al.*¹⁵ with permission from Wiley) illustrates the principal result of this test which is simply the number of marbles buried. Clearly, the TPH2 KO mice buried significantly more marbles than their WT counterparts (13 versus 4 out of 20, respectively). Perhaps the one challenge in scoring this test is the definition of a buried marble. In this test, a marble is scored as buried if two-thirds of its surface is covered by bedding¹⁰. WT mice bury few marbles, so scoring them is generally not a problem. However, we find that TPH2 KO mice not only bury marbles but move large amounts of bedding around the cage as they burrow and dig, making scoring a greater challenge. Because this scoring is somewhat subjective, we use 2-3 independent scorers who are blind to the genotype or treatment of the test mice. As long as marbles are not accidentally displaced when test subjects are removed from the cage, the marble burying test is a robust and sensitive method for the analysis of repetitive, compulsive-like behaviors in normal mice and in mice that have been genetically modified. The usefulness of this test for assessing the effects of numerous classes of drugs on repetitive behaviors has also been established^{6,7,10}.

Nestlet shredding is another test that takes advantage of the natural proclivity of mice to shred practically any material for nest construction. Males and female mice are both reliable shredders. As in the marble burying test, a single investigator can set up large numbers of test cages and then leave the test room for the duration of the test session. **Figures 3A** and **3B** show two cages from WT mice after conclusion of the test. The nestlets in **Figures 3A** and **3B** were 11% and 27% shredded, respectively. **Figures 3C** and **3D** show representative nestlets from two TPH2 KO mice and show that nestlets are much more extensively shredded (*i.e.* 51% and 100% shredded in **Figures 3C** and **3D**, respectively). Because nestlets are carefully weighted before placement into the cage, the analytical task in this test is to weigh the remainder of the intact nestlet after test completion to quantify the amount of nestlet shredded. In our experience, two issues can slightly complicate weighing of the nestlets after shredding. First, if the nestlets are even slightly moist, the weight of the remaining nestlet will be artificially high. To avoid this situation, we allow shredded nestlets to dry O/N to remove any moisture and we withhold food and water bottles (as sources of moisture) from cages during the test. Second, the shredded nestlets are not always as "cleanly" or totally shredded as in **Figure 3D**. It can be seen that some nestlets contain adherent material that has clearly been shredded from the main body of the nestlet, but is still clinging to the intact part. As in the marble burying test, we use 2-3 raters to judge the amount of adherent but shredded material that should not be considered part of the remaining intact nestlet. This material is then carefully teased away from the intact nestlet with forceps and the remaining unshredded portion is weighed. An example of this condition is presented in the inset to **Figure 3A** with arrows pointing to material that should be removed prior to weighing of the remaining nestlet. **Figure 4** (reprinted from Angoa-Pérez *et al.*¹⁵ with permission from Wiley) illustrates a representative outcome of nestlet shredding when comparing WT mice to TPH2 KO mice. It can be seen that TPH2 KO mice show significantly more shredding than WT controls (~50% versus 10% shredded, respectfully). The nestlet shredding test is a simple yet robust and sensitive method for assaying repetitive, compulsive-like behaviors in mice. Nestlet shredding is also a good compliment to the marble burying test and its effectiveness in testing a wide variety of drugs for their effects on repetitive behaviors has been well established¹⁰.

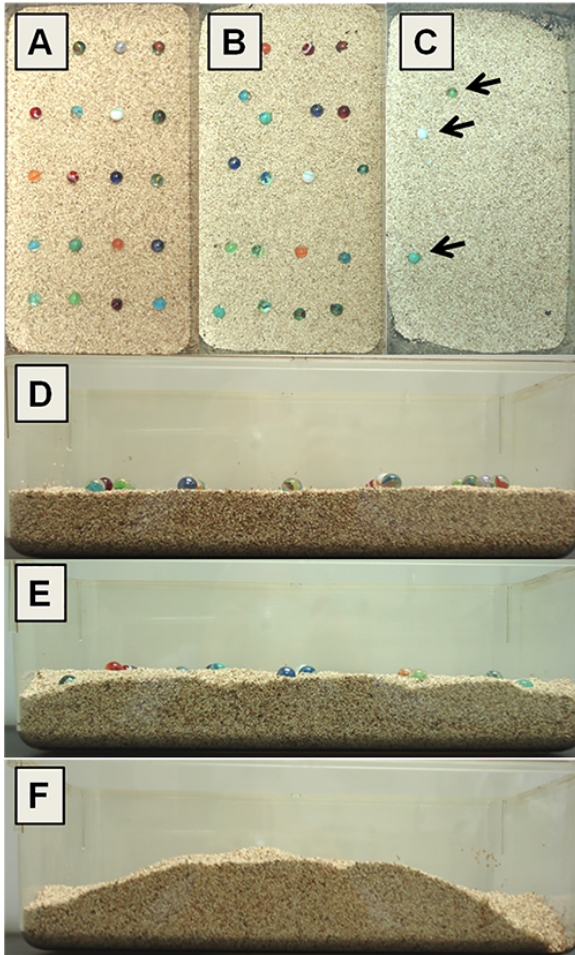


Figure 1. These photographs show representative cages before and after the marble burying test using WT and TPH2 KO mice as subjects. Panels **A-C** are photographed from above the cages and panels **D-F** are photographed along the surface of each cage. **A)** An example of a typical cage prior to introduction of a test subject, showing initial marble alignment; **B)** An example of a representative cage from a WT mouse after burying. In this example, few marbles are buried ($N = 3$) or displaced from their initial location; **C)** An example of a typical cage after testing a TPH2 KO mouse showing that 17 of the 20 marbles were buried in this case. Unburied marbles are indicated with arrows; **D)** A view of the bedding surface before introduction of a test mouse; **E)** An example of alterations in the bedding surface after removal of the WT mouse and **F)** An example of the bedding surface after testing a TPH2 KO mouse. The photograph in panel **F** cannot fully demonstrate in two-dimensions the disruption of the bedding material caused by the TPH2 KO mouse. Not only have most marbles been buried, the surface of the bedding appears to have been excavated by the TPH2 KO mouse. Areas of large troughs and peaks are clearly visible and it is typically the case that mice will dig and displace all bedding from one corner of the cage. [Click here to view larger image.](#)

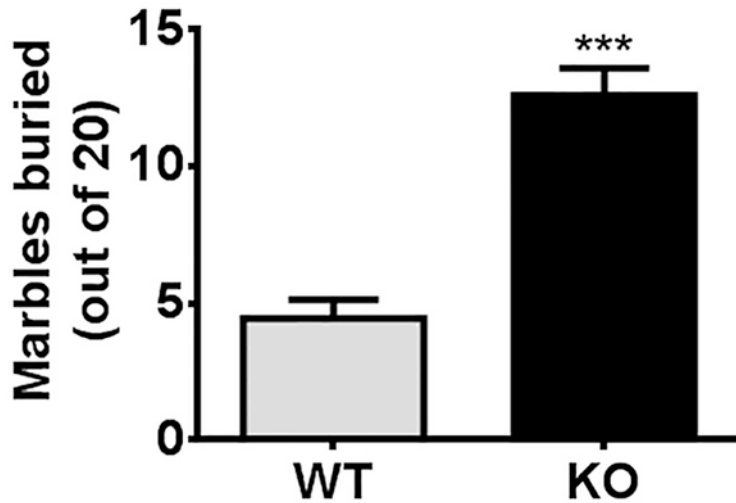


Figure 2. This graph shows typical performance of WT and TPH2 KO mice on the marble burying test. Observers blind to the genotype of test mice score each cage for the number of marbles buried (*i.e.* two-thirds of their surface covered with bedding). The scores for each test subject are averaged for all scorers and plotted as number of marbles buried versus mouse genotype. TPH2 KO mice (N= 20; 10 males and 10 females) bury significantly (two-way ANOVA, $F_{1,66} = 35.61$, $p < 0.05$; comparing sex and genotype) more marbles than WT mice (N= 37; 26 males and 11 females). Males and females did not differ in marble burying so data was combined across this variable within each genotype. Results are mean $\pm$ standard error of the mean. *** indicates $p < 0.001$ for Bonferroni *post hoc* comparisons between WT and KO mice. This figure is reprinted from Angoa-Pérez *et al.*¹⁵ with the permission of Wiley. [Click here to view larger image.](#)

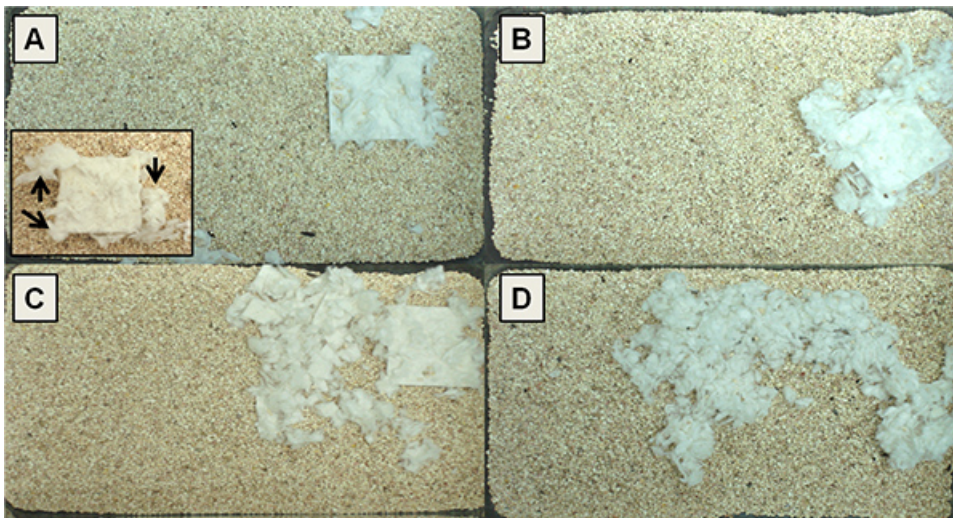


Figure 3. These photographs show representative nestlets after shredding. **A)** and **B)** Examples of nestlets from 2 male WT mice showing minor amounts of shredding (*i.e.* 11% and 27% shredded in A and B, respectively, in these examples); **C)** and **D)** Examples of nestlets from 2 male TPH2 KO mice showing greater amounts of shredding (51% and 100% shredded in C and D, respectively, in these examples). Note in some cases that shredded material remains adherent to the main body of the unshredded nestlet (see inset to panel A). We use 2-3 scorers to judge the amount of shredded material that can be removed from the unshredded remainder of the nestlet. After this material is removed carefully, the remaining unshredded nestlet is weighed. [Click here to view larger image.](#)

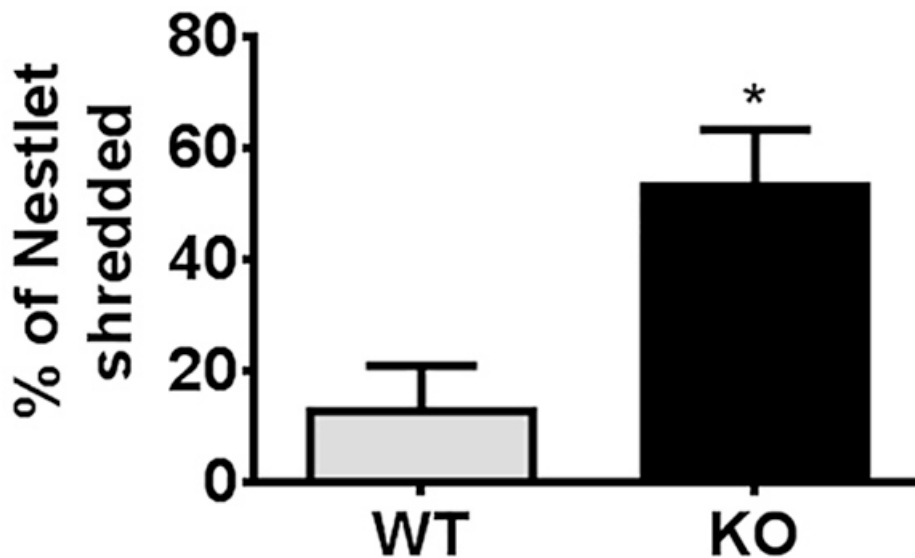


Figure 4. This graph shows typical performance of WT and TPH2 KO mice on the nestlet shredding test. The weight of the remaining unshredded nestlet is divided by its initial weight to calculate the percentage of nestlet shredded and plotted versus mouse genotype. The TPH2 KO mice (N = 6 male mice) shred significantly (Student's *t*-test, $t_{10} = 3.07$, $p < 0.05$) more nestlet material than WT mice (N = 6 male mice). Results are mean $\pm$ standard error of the mean. * indicates $p < 0.05$. This figure is reprinted from Angoa-Pérez *et al.*¹⁵ with the permission of Wiley. [Click here to view larger image.](#)

Discussion

The marble burying and nestlet shredding tests are good examples of behavioral methods for studying repetitive and compulsive-like behaviors in mice. Both tests show excellent face, construct and predictive validity for the human disorders they model⁶⁻¹¹. These repetitive behaviors are natural and spontaneous in rodents and their frequency and intensity can vary after genetic modifications, as shown presently, or after head injury and treatment of subjects with drugs. The value of these two tests derives from the fact that they are validated models of human psychiatric disorders such as OCD and ASD, both of which involve expression of repetitive, unwanted behaviors. Both tests are robust and sensitive to large variations in the expression of digging, burying and shredding behaviors in mice, as illustrated by TPH2 KO mice.

With respect to existing methods (e.g. genetic, pharmacological, and neurodevelopment models) the marble burying and nestlet shredding tests have numerous advantages. Both are readily available to most labs without the need for sophisticated and expensive equipment. These tests are simple to apply, they can be formatted for high-throughput analyses and they are easy to score reliably. With slight modifications, these models can provide additional valuable information that can be useful when studying more complex behaviors and disorders. For instance, test sessions in both models can be video-taped and mice can be scored for locomotor activity, distance traveled, time spent moving, and percent of time spent in each region of the test cage (e.g. center versus perimeter). Measures such as these can provide revealing information on levels of anxiety, particularly if using subject mice with genetic manipulations that might be anxiogenic. Drug treatments can also modify repetitive behaviors indirectly by altering locomotor activity, so the parallel measure of this parameter along with marble burying or nestlet shredding could reveal drug-induced effects on movement.

One limitation of the marble burying test relates to the question of whether marble burying actually represents compulsive-like behavior or if the marbles evoke novelty-induced or anxiety-like responses in mice. Careful study has shown that the glass marbles do not serve as a fear- or anxiety-producing stimulus^{19,20} and attempts to habituate mice to the marbles do not change burying. It could also be argued that marble burying is actually secondary to digging and burrowing in rodents. Therefore, mice do not bury marbles specifically but in the process of digging and burrowing, marbles become covered by the bedding. This potential limitation can be tempered by the observation that digging correlates with burying and not with other behaviors emitted during this test¹⁹⁻²¹. Another limitation of marble burying is under-estimation of the behavior in animals that show excessive amounts of digging and burrowing (e.g. TPH2 KO mice). These mice dig intensely in the absence of any objects in the test cage¹⁶ and a similar situation could occur in other genetically modified mice or in subjects treated with certain drugs. Thus, it is likely that marbles are buried and subsequently uncovered, to be scored inaccurately as unburied. Finally, a limitation that applies to both the marble burying and nestlet shredding tests is the fact that mice will often climb on and hang from the wire cage tops. This competing behavior will diminish the amount of time spent in marble burying and nestlet shredding. This climbing behavior is natural in rodents and could represent a repetitive behavior as well. However, to avoid this potential problem, flat wire tops or filter-top covers can be used.

Modifications in the marble burying test can help overcome these possible limitations. First, test sessions can be video-captured but this modification would involve added work in post-session video review and analysis. Second, marbles and their initial locations can be marked individually so that their movement can be tracked after session completion. Third, analysis of the topographic changes in the bedding surface (i.e. size and number of troughs and peaks in bedding) can be assessed and applied as an adjunct to time spent digging.

A critical step for both protocols is the use of fresh bedding to minimize the influence of interfering olfactory cues from subject to subject. Marbles should be cleaned carefully before each test and marbles and nestlets should always be handled with gloves for the same reason. To the extent possible, flat wire cage covers or filter-top covers should be used with deeper cages so that mice cannot reach the wire covers and spend time

climbing. In addition, cages should have a minimum size of 26 cm x 48 cm x 20 cm to allow even dispersion of the marbles so that mice can ambulate in the cage without stepping on the marbles. At the completion of marble burying sessions, mice must be removed carefully to avoid movement of the marbles prior to scoring. After the completion of the nestlet shredding test, it is critical to allow nestlets to dry before their post-session weights are measured to avoid under-estimation of shredding. Finally, both tests should be scored by observers blinded to any variables or treatment applied to the subjects under study (e.g. genotype of mice being compared, drug treatment).

In summary, the marble burying and nestlet shredding tests provide accurate and sensitive assays of repetitive and compulsive-like behaviors in rodents. Future applications should extend these tests to the screening of new treatments for human conditions such as OCD and ASDs. These tests would also be valuable in the assessment of repetitive behaviors after traumatic brain injury and exposure of animals to chronic stress because each of these conditions can engender compulsive-like behaviors in humans and animals alike.

Disclosures

The authors declare that they have no competing financial interests.

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