

Buried Food-seeking Test for the Assessment of Olfactory Detection in Mice

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[Abstract] The sense of smell allows animals to discriminate a large number of volatile environmental chemicals. Such chemical signaling modulates the behavior of several species that depend on odorant compounds to locate food, recognize territory, predators, and toxic compounds. Olfaction also plays a role in mate choice, mother-infant recognition, and social interaction among members of a group. A key assay to assess the ability to smell odorants is the buried food-seeking test, which checks whether the food-deprived mice can find the food pellet hidden beneath the bedding in the animal's cage. The main parameter observed in this test is the latency to uncover a small piece of chow, cookie, or other pleasant food, hidden beneath a layer of cage bedding, within a limited amount of time. It is understood that food-restricted mice which fail to use odor cues to locate food within a given time period are likely to have deficits in olfactory abilities. Investigators who used the buried food test, or versions of the buried food test, demonstrated that it is possible to evaluate olfactory deficits in different models of murine studies (Alberts and Galef, 1971; Belluscio *et al.*, 1998; Luo *et al.*, 2002; Li *et al.*, 2013). We have recently used this assay to demonstrate that olfactory-specific Ric-8B knock-out mice (a guanine nucleotide exchange factor that interacts with olfactory-specific G-protein) show an impaired sense of smell (Machado *et al.*, 2017). Here we describe the protocol of the buried food-seeking test, as adopted in our assays.

Keywords Buried food-seeking test, Ric-8B knock-out mice, Olfactory behavior tests, Olfactory impairment, Olfactory sensory neuron

[Background] The buried food-seeking test was first described in 1971 (Alberts and Galef, 1971). Since then, additional versions of the test have been described. This test has been used to investigate the consequences of olfactory impairment in a variety of situations, such as: analysis of the effects of olfactory function on the performance of female mice in social behavior towards male conspecifics (Yamada *et al.*, 2001), the discrimination of the participation of both the main olfactory system and the vomeronasal organ in behavior (Del Punta *et al.*, 2002) or in animal models of hyposulphataemia, a disturbance in sulphate metabolism (Dawson *et al.*, 2005). It was also used to assess sociability and cognitive function in neuronal cell adhesion molecule (Nrcam) null mice (Moy *et al.*, 2009), to study the effects of the selective non-imidazole histamine H₃ receptor antagonist in anxiety and depression-like disorders (Bahi *et al.*, 2014), to analyze the role of endocannabinoids in olfactory sensory neurons (Hutch *et al.*, 2015), and others. There are variations in the buried food test methods used in these studies. For example, some authors used pre-test acclimation in the testing cage to reduce novelty-

induced exploratory activity during the olfaction test, while others did not. It is important to note that this acclimation can help in decreasing response variability within groups. In our previous work, we used the buried food test, in association with other motivational, behavioral, and cellular tests, to determinate whether the sense of smell is impaired in olfactory-specific Ric-8B knock-out mice (Machado *et al.*, 2017). The present manuscript describes the protocol of the buried food-seeking test as adopted in our previous assays, in order to observe aspects of olfactory deficits in mice.

Materials and Reagents

1. Mice should be at least 8-week-old

Notes:

- a. We used 8-week-old C57BL/6J background mice of both sexes, it is important that the animals have the same age.*
- b. It is possible to use this protocol with other strains of mice. It might be applicable to rats as well, but needs standardization of procedures, such as: size of the cage, depth of the bedding, size of the chow pellet, as well as food deprivation time.*
- c. In females, estrus can effect olfactory discrimination. Considering this, we initially did statistical analysis in separating genders: wild-type (4 males and 5 females), heterozygote (3 males and 3 females) and conditional knock-out mice (4 males and 5 females). However, we observed no differences between genders, so in our final results, we used both genders in all groups. We recommend evaluating the differences between genders before deciding to use mixed-gender groups or not.*

2. Filtered and autoclaved water
3. Chow pellets (Food stimulus)

Notes:

- a. We used a 2 g pellet of the same chow the animals were regularly fed with.*
- b. We used AIN-93G chow (for use during rapid growth, pregnancy and lactation, from Rhoister, described in Reeves *et al.* (1993), because it was the chow type regularly fed to our animals.*
- c. Other types of pleasant foods have been previously used as stimulus, such as: Oreo cookie, Kellogg's Fruit Loops, chow covered in peanut butter and other. We found, however, that Kellogg's Fruit Loops did not stimulate foraging through olfactory cues in the mice. In our experiments, regular food chow pellets showed better results in foraging behavior than the Fruit Loops. This may be due to novelty induced hypophagia, so we recommend the use of food stimulus that the animals are accustomed to in order to avoid novelty induced hypophagia.*

Equipment

1. Experimental animal room

Note: An adequate procedure room is essential for the successful development of the behavior test, since it is sensitive to external distractions. This requirement should be carefully considered during the planning stages. An adequate procedure room consists of an isolated and silent experimentation room, where it is possible to avoid the entrance of people during the test.

2. Clean mouse cage of a regular size (30.5 cm length x 16 cm width x 16 cm height or similar) (Figure 1) (We used the M.I.C.E.[®] Animal Care Systems cage [Animal Care Systems, catalog number: M/P 79010])

Note: Do not use regular cage lids. The stainless-steel top which holds food and water interferes with observation and should not be included in the setup. If needed, it is best to use acrylic lids.

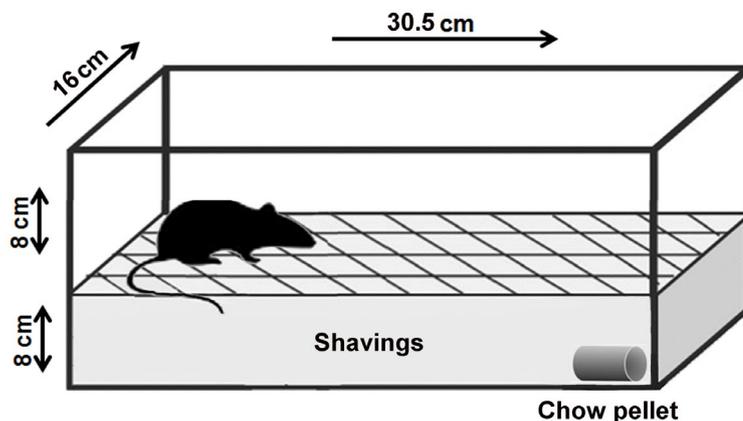


Figure 1. Layout of the buried food-seeking test. The purpose of this experimental test is to measure the animals ability to use olfactory cues for foraging. The main parameter measured in this test is the latency to find the hidden food. Latency is defined as the time between when the mouse was placed in the cage and when the mouse uncovered the food pellet. For the test, 8-week-old mice were deprived of food for 24 h but received water *ad libitum*. Next day, a 2 g pellet of regular chow was buried 8 cm beneath the surface of the fresh bedding in one end of a clean test cage. The site of animal placement and the site at which the pellet was buried remained constant.

3. Fresh cage bedding to create an 8 cm layer in each cage

Notes:

- For each subject, a clean cage washed and dried by the animal care cage washing facility and clean bedding should be used. Do not re-use cages or bedding.*
- Cage lining: we use Premium Hygienic Animal Bedding LIGNOCEL[®] FS-14 to bury the pellet.*

4. Portable digital scale (Denver Instrument, model: TR-403, or equivalent scale)

Note: To weigh the pellet chow, we used a digital scale (Denver Instrument, TR-403, Max = 410

g, d = 0.001g).

5. Digital stopwatch

Note: The stopwatch is used to monitor the test length.

6. Digital video camera (optional, we used Sony Cyber-shot 14.1 mega pixels) (Sony, model: DSC-W330)

Notes:

- a. The presence of a human observer may influence animal behavior. The use of a video camera may help reduce human interference during the test.*
- b. All videos were recorded in .avi format and viewed using VLC media player.*

Procedure

1. Deprive 8-week-old mice of food for 24 h: 24 h before the test, remove all chow pellets from the home cage. Check inside the cage to remove any pellet fragments that may be scattered within the cage. Do not remove the water bottle.

Note: During standardization, we tried using 6-week-old mice and had ambiguous results. 8-week-old mice or older should be used for the behavioral tests because 6-week-old mice are still considered juvenile and show a larger variability in behaviors.

2. On day 2, it is advisable to acclimate the mouse to the room in the testing cage in the same conditions of the test, before the insertion of the pellet. Acclimation should last as long as the test will last and the animal should be taken out of the cage before the insertion of the pellet.
3. Initiate the test by burying a 2 g pellet of regular chow 8 cm beneath the surface of the fresh bedding in one of the corners of the test cage. Transfer the mouse from its home cage back into the test cage, in the opposite corner in regard to the pellet. The time from its introduction into the test cage until it finds the food pellet is recorded as the latency (Video 1). The digital stopwatch should be halted as soon as the mouse touches the pellet of chow. If an animal is not able to find the food pellet within 10 min, the test is terminated and the latency is recorded as 600 sec. Importantly, for each subject, a clean cage, clean bedding, and new pellet of chow should be used.



Video 1. Example of a wild-type mouse foraging during the test. The chow pellet is located in the right corner. The digital stopwatch is halted as soon as the mouse touches the pellet.

4. It is important to control for motivation in food-seeking behavior. This can be done with either the unburied food-seeking test (Li *et al.*, 2013) or other motivation and locomotion tests (Machado *et al.*, 2017). The unburied food-seeking test is the simplest way to control for motivation. It can be done using either parallel control groups (groups that undergo the unburied food-seeking test at the same time that the test groups undergo the buried food-seeking test) or the same animals used in the buried food-seeking test, after one week. The unburied food seeking test is the same as the buried food-seeking test, with exception to the pellet being on the surface of the bedding, instead of buried. Therefore, this test does not depend on olfactory cues and controls for motivation level among groups.

Notes:

- a. In the eventuality of an animal escaping the cage during or immediately before the test, exclude this animal from the experiment.
- b. In Machado *et al.* (2017), while testing for behavioral differences through olfactory impairment, three groups were used: the test-group (conditional knock-out, $n = 9$) and two control groups (wild-type, $n = 9$; and heterozygote mice, $n = 6$). The test-group showed longer latency in retrieving the food pellet, in comparison to the control-groups (WT 143.1 ± 30.2 sec; Het 188.3 ± 24.5 sec; cKO 378.5 ± 76.3 sec; $p = 0.02$, $F_{(2,15)} = 4.54$, one-way ANOVA and Dunnett post-test). It is important to note that in this article, we acclimatized the mice to the experimentation room, but not to the cage. It is possible that further acclimation to the cage may render more intensely different latencies between groups (Figure 2). Additionally, in this study we did not perform the unburied food-seeking test because we evaluated motivation and locomotion through the open-field, light/dark, elevated plus-maze, and tail suspension tests.
- c. In order to evaluate possible differences between male and female mice, the three groups used in Machado *et al.* (2017) were initially divided in genders: wild-type (4 males and 5 females), heterozygote (3 males and 3 females) and conditional knock-out mice (4 males

and 5 females). However, after initial statistical analysis, we observed no differences between genders, so the final results contain both genders in each group.

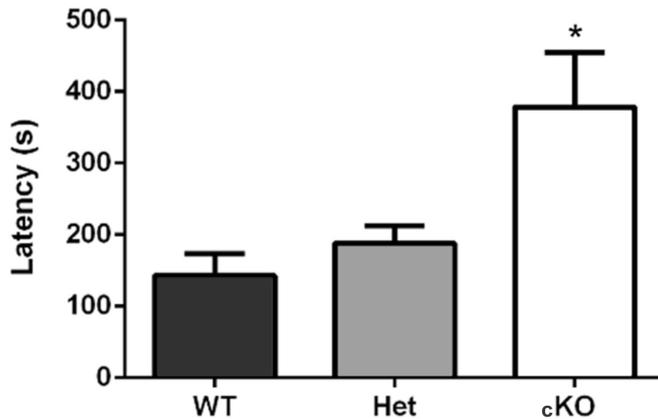


Figure 2. Buried food-seeking test results. Ric-8b conditional knock-out (cKO) mice required significantly longer times to find the food. WT (n = 9), Het (n = 6), and cKO (n = 9) (extracted from Machado *et al.*, 2017).

Data analysis

1. For experimental design, at least three animals of each group test should be used.
2. The time necessary for the animal to retrieve the pellet (latency) was measured in seconds up to a maximum of 10 min (600 sec was the maximum score).
3. Any preferred statistical program can be used for statistical analysis. To compare two data sets, use Student's *t*-test. To compare three data sets or more, use One-way ANOVA and *post-hoc* Dunnett or similar. For not normal distribution of data or small groups, use Mann-Whitney test to replace the Student's *t*-test and Kruskal-Wallis in replace for one-way ANOVA. Differences are considered significant at $P \leq 0.05$.

Notes

1. The score on a behavioral test can be influenced by many factors, such as lab temperature, noise and the animal's circadian rhythm. In this way, the experiments should always be done at the same time of day, always respecting the animals circadian cycle, in an isolated and silent experimentation room, avoiding the entrance of people during the test. It is highly recommended to acclimate the animals in a separate room dedicated specifically for acclimation; this room should be similar to the testing room.
2. A single experimenter should complete an experiment in its entirety, to reduce variability. Importantly, if multiple cohorts of mice are used, it is preferable that the same experimenter conduct testing on all the mice in all the cohorts of the study to reduce variability.

3. We recommend double-blind tests. The experimenter that does the video analysis should be blind to the experimental groups in order to avoid bias.

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Competing interests

The authors declare no competing financial interests.

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