

Video Article

An Objective and Reproducible Test of Olfactory Learning and Discrimination in Mice

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Abstract

Olfaction is the predominant sensory modality in mice and influences many important behaviors, including foraging, predator detection, mating, and parenting. Importantly, mice can be trained to associate novel odors with specific behavioral responses to provide insight into olfactory circuit function. This protocol details the procedure for training mice on a Go/No-Go operant learning task. In this approach, mice are trained on hundreds of automated trials daily for 2–4 weeks and can then be tested on novel Go/No-Go odor pairs to assess olfactory discrimination, or be used for studies on how odor learning alters the structure or function of the olfactory circuit. Additionally, the mouse olfactory bulb (OB) features ongoing integration of adult-born neurons. Interestingly, olfactory learning increases both the survival and synaptic connections of these adult-born neurons. Therefore, this protocol can be combined with other biochemical, electrophysiological, and imaging techniques to study learning and activity-dependent factors that mediate neuronal survival and plasticity.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57142/>

Introduction

The mouse OB, where odor information first enters the central nervous system (CNS), provides an excellent model to study experience-dependent structural changes. OB circuitry continually integrates adult-born neurons in an activity-dependent manner. Adult-born neuron precursors divide off from progenitors that line the subventricular zone adjacent to the lateral ventricles¹. Upon migrating into the OB, these neuronal precursors either survive, differentiate, and integrate as inhibitory granule cells, or undergo apoptosis². Selection for cell fate is influenced by olfactory activity, including olfactory learning^{3,4,5,6}. After integration, learning-induced synaptic changes occur in granule cells during a two-week critical period^{7,8}. Thus, assays for olfactory learning are useful for examining how experience-dependent plasticity influences structural and functional reorganization of a mature brain circuit.

This protocol offers one approach to olfactory training by using an operant conditioning paradigm. In this task, water-deprived mice are trained to associate one odor (the "Go" odor) with a water reward and another odor (the "No-Go" odor) with a trial timeout punishment. Mice progress through a graded series of training phases over the course of 2–4 weeks. When training is complete, mice respond to the Go or No-Go odor with discrete, corresponding behaviors (seeking a water reward on Go trials and not seeking the water reward on No-Go trials) (**Figure 1A**). After training is complete, mice can be further challenged with chemically similar odor pairs to test discrimination or become transitioned to studies investigating how olfactory learning alters the structure or function of the OB. Although odor discrimination tasks have been previously described, most rely on subjective measurements such as number of sniffs between two odorants^{9,10}. Furthermore, the need for human scoring of such tasks is also time-intensive. The Go/No-Go olfactory learning task described in this protocol offers an unbiased, direct measurement of odor discrimination and olfactory learning.

Protocol

All mice were used under a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee in accordance with NIH standards. Mice used in this protocol were all adult mice (>6 weeks of age) on C57BL6/j background and included both male and female mice. After training/staging tasks, mice are returned to their home cage.

1. Construction and General Rules for Using Operant Learning Box (Figure 1B, C)

1. Assemble a mouse chamber with chamber floor. Keep the training box in a low traffic, dimly lit area to avoid distractions.
2. Drill each water port with a small hole on top to allow an 18-gauge needle to dispense water inside the port.
3. Fill glass vial with odorant dissolved in mineral oil (replaced weekly), and securely tighten the cap.
4. Connect 18-gauge needle to silicone tubing. Pierce glass vial cap with 18-gauge needle, and connect other end of silicone tubing to the intake of the odor ports.
5. Place each silicone tubing into an odor valve.
6. Connect the vacuum line to the odor ports.
7. Attach two 10 mL syringes to a metal holding rod, and connect tubing to the syringes. Connect the other side of the tubing to an 18 G needle. Fit the needle into the drilled hole of the nose poke port on the mouse chamber. Connect the other end of the tubing to the water valve.
8. Fill the two 10 mL syringes with rodent drinking water.
9. Connect an air-flow meter to the air intake and maintain air flow at 3–5 L/min.
10. Connect the 2 water valves, 2 odor valves, 2 water ports, odor port, and power to the USB interface system. Connect all valves to 'Output' ports, all the odor and water ports to 'Input' ports. Connect all equipment to power outputs from the USB interface box, and lastly, connect the USB interface box to power.
11. Adjust the vacuum suction to avoid cross contamination of odors between trials.
12. Use odor-specific tubing to connect odorant vials to the chamber.

2. Mouse Preparation: 1–3 days

1. Divide mice into 3 groups: control (no olfactory training necessary), pseudo-trained (mice who receive reward or punishment at random), and trained groups. Expose pseudo-trained mice to the training box and odor delivery, but do not provide olfactory training because the outcome of reward versus punishment is randomly associated with the odor delivered.
NOTE: The pseudo-trained group will go through the training paradigm under the "Pseudotraining" stages. Trained mice will complete all training stages. Pseudotraining is optional if the purpose of the experiment is to assay for behavioral differences in odor discrimination or learning. The protocol provided here adds this group if the experimenter wants to probe for neuronal differences before and after training. The pseudotrained group would then control for passive odor exposure and non-olfactory related training.
2. Begin water restriction in mice to 40 mL/kg/day. Avoid bodyweight loss greater than 20% of the animals' baseline weight in order to avoid distress (Figure 2A).
3. Weigh the mice daily to ensure they are above 80% of baseline weight. If a mouse falls below this threshold, remove the mouse from the study and provide free access to water.
4. Keep all environmental factors constant throughout the protocol including temperature, noise, and stray odors (including personal body and perfume/cologne/deodorant scents).
NOTE: As with all animal behavior testing, small environmental changes can greatly influence results.

3. Instructions for All Stages

1. Code training software for each stage below. Run the software on the behavioral software.
NOTE: Coding for all stages are contained in supplemental coding files. Data for 20 trials are grouped as a single block and mouse performance is displayed across blocks. Furthermore, each stage can be repeated on a mouse for a number of days until completion criteria are met.
2. Do not keep mice in the behavior box for more than 60 min/day.
3. Clean mouse waste from the cage before each mouse is transferred to the cage. Spray and wipe the chamber with 70% ethanol to minimize mouse odor distraction.

4. Training Stage 1: Associating Water Reward with a Center Nose Poke, 1–3 Days

1. In this stage, associate mice with a water reward upon exploration of the water port.
2. **Training Stage 1 programming instructions**
 1. Program this stage to only use the water delivery port. Let the mouse receive a water reward for each nose poke.
NOTE: The program will output the time duration of the trial and the total number of water rewards the mouse received.
3. Set the pseudotraining Stage 1 programming the same as Training Stage 1.
4. **Box configuration and mouse setup**
 1. Configure the behavior box with a water port in the center and with all side ports inaccessible. Place a mouse into the chamber. Close the mouse chamber and begin the Stage 1 program.
5. Consider this stage as complete when the mouse achieves 100 trials within 60 min. Remove the mouse from the chamber after 60 min or 100 trials have been completed (Figure 2B).
NOTE: Due to individual differences, some mice will naturally refrain from exploring the box.
6. If encouragement is needed, manually deliver water into the water port. Repeat this stage for up to 3 days.
NOTE: Mice that are already trained can be repeated further to maintain parity and to keep them on fluid restriction. It is also possible to promote an entire group to stage 2 when the group average reaches 100 trials/60 min. This will allow for all mice to continue training on the same day.

5. Training Stage 2: Associating a Side Port Water Reward with Center Port Nose Poke, 1–5 Days

1. In this stage, let the mice poke their nose in the center port and then immediately receive a water reward on the side ports.
2. **Box configuration and mouse setup**
 1. Configure this and every subsequent stage with 2 water ports on the sides and the odor port in the middle. Place a mouse into the chamber. Close the mouse chamber and begin the Stage 2 program.
3. **Training Stage 2 programming instructions**
 1. Provide the mouse with an immediate water reward on both sides after a nose poke into the center odor delivery port. Set output parameters for this stage as the time duration of the trial, the number of trials initiated, and the number of water rewards received within 5 s of a nose poke.
4. Set the pseudotraining Stage 2 programming the same as Training Stage 2.
5. Consider this stage as complete when a minimum of 40 trials are performed in 60 min, with at least 25% of water rewards received within 5 s of the center port nose poke. Remove the mouse once this stage has completed (**Figure 2C**).
 NOTE: The training program calculates the percentage of water rewards received in a timely manner in order to determine completion of this stage. Although mice vary in how fast they complete this stage, most mice will reach completion criteria within 5 days. However, if a mouse has not completed this stage within 5 days, do not advance the mouse to the next stage. This mouse will be removed from the cohort.

6. Training Stage 3: Associating a Water Reward with a Specific Odor and Within a Specific Time Window, 1–3 Days

1. In this stage, let the mice receive a Go (S+) odor upon a nose poke in the center port. Subsequently, yield a water reward upon a nose poke into the side water ports within 5 s.
2. **Training Stage 3 programming instructions**
 1. Deliver the S+ odor in the center odor port.
 2. Deliver the water reward if the mouse pokes the side ports within 5 s of an odor delivery.
 NOTE: The program begins by only requiring a 100 ms nose poke in the center port to yield a water reward. Once the mouse pokes the center port for correct amount of time in 80% of trials, the time duration of a nose poke required for a water reward will increase by 50 ms up to 400 ms.
 3. Set the output parameters identical to stage 2.
3. Set the pseudotraining Stage 3 programming instructions the same as Training Stage 3. However, do not connect the S+ odor to the odor delivery controller to ensure that the S+ odor is not associated with a water reward.
4. **Box configuration and mouse setup**
 1. Set the same configuration at stage 2.
 2. Connect the S+ odor to the odor delivery controller. Place a mouse into the chamber. Close the mouse chamber and begin the Stage 3 program. Remove the mouse once this stage has completed.
5. Consider this stage as complete when there are greater than 60 rewards within 60 min (**Figure 2D**).

7. Stage 4A: Associating No-Go (S-) Odor and Time-out Punishment, 1–2 Days

1. In this stage, introduce the mice to a No-Go odor (S-). Provide a time out punishment to the mice that incorrectly go to the water ports after smelling the no-go odor.
2. **Training Stage 4A programming instructions**
 1. Deliver only the S+ odorant in this stage in the beginning, identical to stage 3. After mice complete 40 trials, begin random delivery of the odors to include both S+ and S- odors. Program a 2-s time out punishment if the mouse attempts to seek a water reward after being presented the No-Go odor.
 NOTE: To aid in distinguishing Go vs. No-Go, the IR side lights can be manipulated such that they are on during Go odorants and off during No-Go odorants. The lights act as a secondary cue to aid mice in initial training. Once mice are trained with the lights, they should under-go training without lights to confirm they have learned the task with odors.
 2. Set the output parameters as time duration of the trial, number of trials initiated, number of trials completed, % correct, and total number of rewards received.
3. Set the pseudotraining Stage 4A programming instructions the same as Training Stage 4S except S+ and S- odors are presented randomly from the start. Give water reward or timeout punishments at random, regardless of task performance.
4. **Box configuration and mouse setup**
 1. Set the box configuration the same as previous stage 3.
 2. Connect both S+ and S- odor to the odor delivery controller. Place a mouse into the chamber. Close the mouse chamber and begin the Stage 4A program. After the mice complete 40 trials, switch the program to randomly deliver the odors. Remove the mouse once this stage has completed.
5. Consider this stage as complete when there are 40 trials with > 60% correct responses.

8. Stage 4B: Associating No-Go (S-) Odor and Time Out Punishment, 5–11 Days

1. Training Stage 4B programming instructions

1. Make this stage identical to stage 4A, however the time out punishment for attempting a water reward after a S- odor is 4 s.

2. Set the pseudotraining Stage 4B programming instructions identical to stage 4A, however water reward and time out punishment are randomly delivered per trial.

3. Box configuration and mouse setup

1. Set the box configuration the same as previous stage 4A.
2. Connect both S+ and S- odor to the odor delivery controller. Place a mouse into the chamber. Close the mouse chamber and begin the Stage 4B program. Consider this stage complete when there are >100 trials within 60 min with accuracy >85% (**Figure 2E**)

4. Monitor the maximum and minimum percentage correct for each session to track each mouse's progress in this stage. Approximately 85-90% of mice reach completion criteria for this stage. Exclude mice that do not achieve completion of this stage for the Go/No-Go testing phase.
5. (Optional) At the end of this stage, apply a reversal training, where the Go/No-Go odorants are switched on the odor vial platform. Ensure that the mice are not associating with another stimulus, such as the sound of the valves associated with the side ports.

9. Go/No-Go Assay Task: 1 Day, 20 Min per Mouse per Day

1. Consider this as the final stage to determine accuracy of identifying odor associations and learning to discriminate odor pairs.

NOTE: Novel odorants are utilized for S+/S- to test how long animals take to learn new associations. Structurally similar odorant pairs are used to increase task difficulty. Examples include 1-Butanol vs. 1-Pentanol, Isoamyl Acetate vs. Isoamyl Butyrate, and + Carvone vs. - Carvone.

2. Programming instructions

1. Let the training software detect the length of nose poke. Program a 300 ms nose poke in the center odor port to execute the task.
2. Set the output parameters identical to stage 4B, except do not use any light guided cues.

3. Box configuration and mouse setup

1. Set the box configuration the same as previous stage 4B.
2. Connect both S+ and S- odor to the odor delivery controller. Place a mouse into the chamber. Close the mouse chamber and begin the Stage Go/No-Go program. Expose the mice that have learned the olfactory learning task to novel pairs of chemically similar odors. NOTE: Wildtype mice with adequate odor discrimination reach >85% accuracy with novel odorants after approximately 10-20 blocks or 200-400 trials (**Figure 3A**).

Representative Results

Once mice have learned the olfactory learning task, they can now associate novel odor pairs with reward and punishment. These trained mice normally begin with about 50% accuracy on the Go/No-Go task. The percentage correct can be plotted by trial block as a learning curve for novel odor pairs (**Figure 3A**). Within 10 block trials, which take most mice less than 30 min to perform, mice are able to correctly discriminate between odors with greater than 85% accuracy (red line). This shows that our protocol has successfully trained wildtype mice to associate one odor with a water reward and another with a timeout punishment. To assess the discrimination capabilities between two cohorts, these data can be further analyzed to compare the number of trials needed to reach 85% proficiency (**Figure 3B**) or by the average percent correct after reaching proficiency.

Once mice learn the task, odor pairs can be modified to increase or decrease the task difficulty. For example, decreasing the odor-pair concentration increases the task difficulty (**Figure 3C**). This analysis can reveal the threshold of detection for different mice cohorts. Furthermore, odor pairs can be changed to become more structurally similar (*i.e.*, enantiomers or single carbon differences). Odor mixtures can also be used to increase task difficulty (*i.e.*, 40/60 mixture vs. 30/70 mixture).

Mice are also able to remember previous learned odor pairs. After waiting 7 days since learning the task, a recall test show that wildtype mice can quickly remember previously learned odor associations (**Figure 3D**).

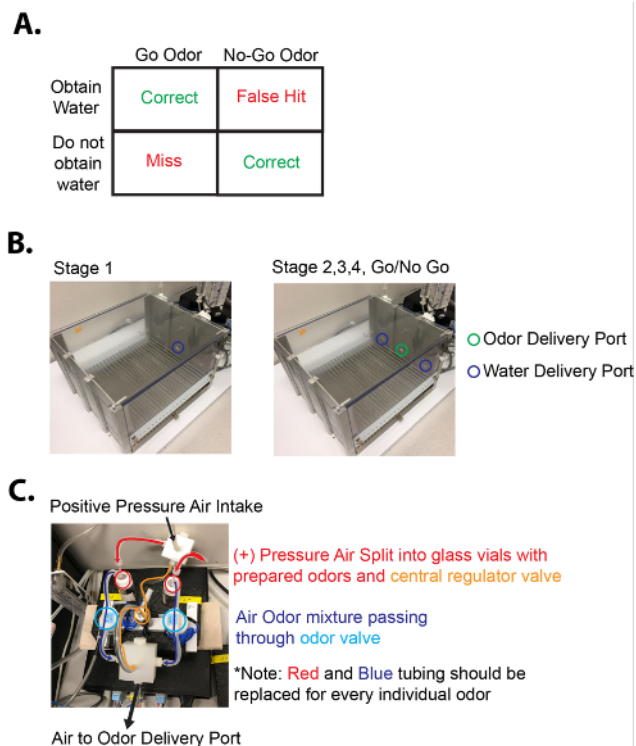


Figure 1: Go/No-Go training paradigm and equipment configuration. (A) Mice respond correctly by either obtaining water during Go Odor presentation or by refraining during No-Go Odor presentation. (B) The behavior box is configured such that only a single water port is accessible during stage 1 and all subsequent stages contain both a central odor delivery port and two water ports. Blue circle: water port. Green circle: odor port. (C) Odor delivery is gated by one odor valve for each odor. Positive pressure air intake leads to a distributor. Red arrows indicate positive air pressure line going into the prepared odor vial (red circle). Blue arrow indicates air/odor mixture leaving the odor vial and passing through an odor valve (light blue circle) and entering a white air integrating box. Orange arrow indicates positive pressure air going to a central regulator valve (orange circle) to the integrating box to allow for positive air pressure to push odor/air mixture from integrator box to the odor delivery port (bottom black arrow). [Please click here to view a larger version of this figure.](#)

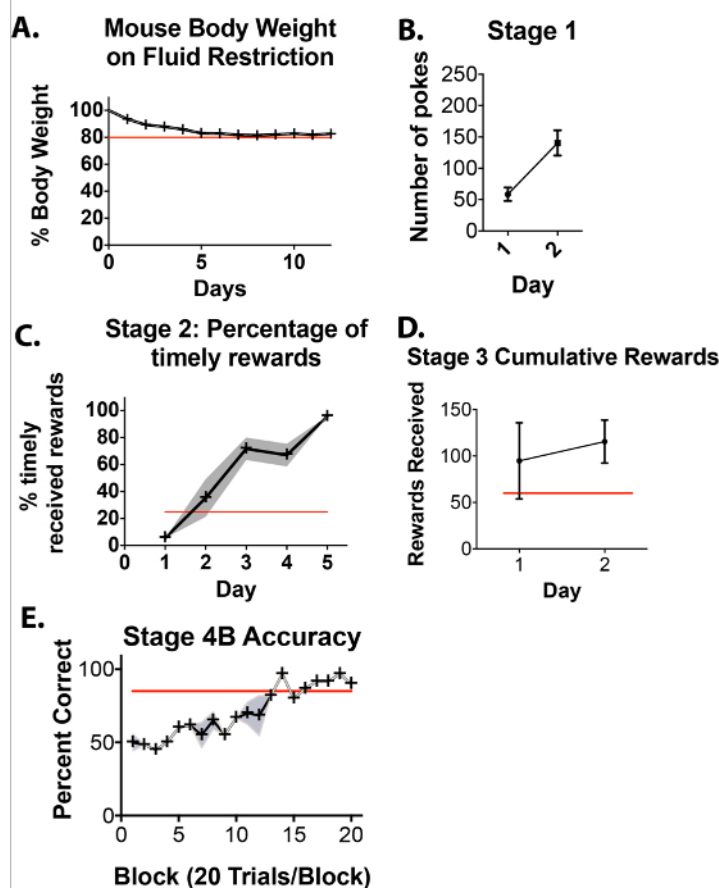


Figure 2: Training data. (A) Mice body weight percentage does not drop below 80% while on fluid restriction. Red line = 80% threshold. N = 8 mice. (B) Stage 1 training results show the number of center port pokes in 60 min to receive water reward. N = 8 mice. Error Bars are standard error of the mean. (C) Stage 2 training results show percentage of water rewards received within 5 s of center nose poke. Red line is 25% threshold. Mean percentage with gray highlighting as standard error of the mean. N = 8 Mice. (D) Stage 3 cumulative rewards show number of rewards received by 8 mice on day 1 and day 2 of training. Red line is 60 reward thresholds. Error bars are standard deviation. (E) Stage 4B accuracy results display percentage correct responses for stage 4B. Red line is 85% of threshold. Solid black line is mean and gray highlighting is standard error of the mean. N = 4 mice. [Please click here to view a larger version of this figure.](#)

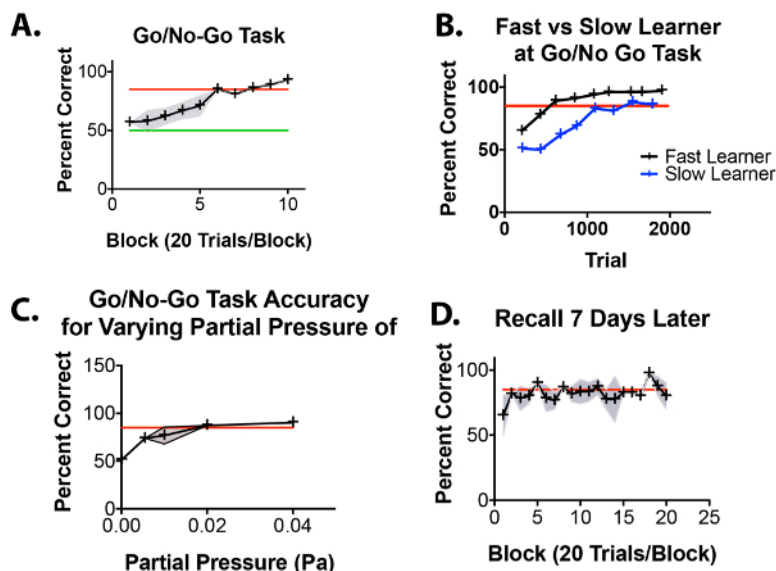


Figure 3: Representative results for Go/No-Go task. (A) Go/No-Go task for 2 novel odorants (S+ = isoamyl acetate/S- = amyl acetate) after mice were trained on eugenol (S+) and eucalyptol (S-). N = 5 mice, with standard error of mean in gray. Green line is 50% accuracy, Red line is threshold 85% accuracy. (B) Example of Go/No-Go task for a mouse that would be a fast learner versus a slow learner mouse for hexanol (S+), and butyric acid (S-). (C) Go/No-Go task for varying partial pressures of hexanol (S+) and butyric acid (S-). (D) Ability of mice to recall the odor pair 7 days after training. [Please click here to view a larger version of this figure.](#)

Discussion

The rodent olfactory system provides a unique model to study sensory dependent plasticity. Here we present an olfactory learning paradigm to train mice to associate odorant pairs with either a reward or punishment. Through this learning task, downstream circuit changes can be studied in subsequent experiments (electrophysiology, *in vivo* neuronal imaging, etc.). Upon completion, mice will learn to perform a simple odor cued task to associate a water reward with one odor, and a timeout punishment with another odor.

Since this is a behavioral assay, we recommend utilizing an equal distribution of age and sex among experimental and control animal groups. It is imperative that the conditions between mice are kept as constant as possible. For example, ensure that the mice handler and lighting conditions remain constant throughout the training. If many mice do not perform the task as expected, ensure the following conditions are met: (1) Keep the behavior setup as quiet as possible. (2) Water deprive the mice sufficiently. We find that even after an entire day of water deprivation, many mice will not sufficiently drink enough from the center port of the stage 1 box to reach completion criteria. (3) At the beginning of each new mouse, check the odor/water valves and tubes to ensure proper placement. (4) Odorants vary in their volatility and therefore some evaporate quicker than others. Replace more volatile odorants with higher vapor pressures daily instead of weekly, especially if lower concentrations are used.

This protocol can be modified according to the experimental purpose. If the learning time in stage 4B is important, then it may be advisable to remove any odors until this stage. This ensures that any olfactory learning begins at stage 4B. To accomplish this, we have performed this protocol by removing the odor from stage 3 and by skipping stage 4A entirely. Mice will have a more difficult time reaching completion criteria using this method, but this also gives valuable information for how quickly mice learn this task the first time.

One limitation of this protocol is that mice have to complete different stages until olfactory tests can be performed. Therefore, although not seen by our lab, a manipulation may impede cognition such that stage 4B is consistently not reached. We have tried to negate this problem by ensuring that each stage is sufficiently long enough to allow most mice to graduate to the next step of training. However, if a mouse does not reach completion criteria for a stage even after the full training period, we remove that mouse from the cohort. This allows us to continue the study without waiting on any individual mouse. Another limitation is that we have yet to extend this protocol to other animal models important for the field of olfaction. Rats, for example, have been instrumental in revealing neuronal function within the OB^{11,12}. Due to their intelligence, rats also have faster learning times than mice^{13,14}. Despite these limitations, we chose mice for this protocol because of their genetic tractability for performing cell type specific manipulations and recordings¹⁵.

Most existing protocols use differences in odor sniffs or time spent next to odors to approximate discrimination or learning^{4,6,9,10,16}. This protocol can directly measure discrimination on a trial by trial basis. Furthermore, this can measure the exact number of trials each mouse needs to learn an association. This fully automated approach removes any human biases from the data analysis. Food deprived animals have also been successfully used for olfactory learning. Water deprived mice were chosen due to the increased number of trials a mouse can perform with water over food¹⁶.

As implantable activity monitors, such as Graded-Index (GRIN) lenses and multi-unit electrodes, continue to improve, we may soon be able to combine those technologies with this protocol^{17,18}. By recording from relevant brain areas during olfactory learning, it is possible to probe how neuronal activities change during associative learning. This may help reveal fundamental neuroscience questions, such as how neurons encode information differently during learning.

Disclosures

The authors declare no conflicts of interest and no competing interests.

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