Video Article

Simple and Computer-assisted Olfactory Testing for Mice

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Abstract

Olfaction is highly conserved among species and is required for reproduction and survival.

In humans, olfaction is also one of the senses that is affected with aging and is a strong predictor of neurodegenerative diseases. Thus, olfaction testing is used as a non-invasive diagnostic method to detect neurological deficits early on. In order to understand the mechanisms underlying olfactory network susceptibility, olfactory research in rodents has gained momentum in the past decade.

Here, we present a very simple, time efficient and reproducible olfactory testing method of innate odor perception and sensitivity in mice without the need of any prior food or water restriction. The tests are performed in a familiar environment to the mice, require only the scents and a 2 min session of odorant exposure. The analysis is performed, *post-hoc*, using computer-assisted commands on ImageJ and can be, therefore, carried out from start to end by one researcher.

This protocol does not require any special hardware or setup and is indicated for any laboratory interested in testing olfactory perception and sensitivity.

Video Link

The video component of this article can be found at http://www.jove.com/video/52944/

Introduction

Olfaction is one of the most developed and important sensory functions in mammals. Any impairment in olfactory activity may affect food intake, social behavior and, in the worst case scenario, even survival. In humans, olfactory deterioration is age dependent[1] and is considered a strong predictor of neurological disorders[2–6]. The olfactory identification test developed by the University of Pennsylvania currently represents one of the most used, non-invasive and quantifiable, diagnostic tests which can assess early neurological deficits[7] and predict with high probability the progression of dementia[8,9].

The accessibility of the olfactory system and the prominence of olfaction in rodents, has sparked an intense line of research addressing the mechanisms underlying olfactory functions[10]. We have previously shown that loss of function of the signaling receptor Notch1 affects olfactory avoidance[11]. In this protocol we use mice lacking the signaling ligand, Jagged1, in neurons or glia to study olfactory performance.

Innate olfaction is defined by three parameters as perception, discrimination between odors and olfactory sensitivity[4]. Olfactory testing in rodents can be done in a variety of ways and some behavioral studies make use of olfactometers, which provide the odor to the animal at a specific vapor concentrations and in a precise time frame[12–14]. Nevertheless, this instrumentation is expensive and may be available only in specialized facilities. In our work, we provide a simple, fast and reproducible olfactory testing protocol, which is carried out using volatile scents. The tests described measure perception to an attractant or a repellent odor and evaluate the discrimination between the scent and water[11,15,16]. Using the same setup, we also can measure the sensitivity to an odor at different concentrations[16,17]. The *post-hoc* computer-assisted video processing, inspired by the work of Page and colleagues[18], provides unbiased results without the need of experimental blinding and allowing for a single person to carry out the whole experiment.

This protocol is intended to provide a starting point for studying olfactory behavior in mice.

Protocol

All the animal procedures are in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes and are approved by the local Animal Care Committee (Canton of Fribourg, Switzerland).

1. Animal Preparation

1. Experimental animals
    1. Perform experiments on adult male wild type and transgenic mice (C57BL/6 background) of 3-5 months of age. The three groups of mice correspond to wild type littermate controls (group A, Jagged1 flox/flox[19]) and two conditional KO mouse lines (group B, Jagged1ncKO and C, Jagged1gcKO).
    2. House mice under standard laboratory conditions in a ventilated room, with a 12 hr controlled dark/light cycle and provide food and water *ad libitum*.

2. Experimental Setup

1. Experimental arena
    1. For the experimental arena, use a clean sterilized mouse cage (36 cm length x 20.5 cm width x 13.5 cm height) (**Figure 1A**).
    2. Assign each mouse to a numbered cage with fresh bedding, 3 cm high. If cages are reused, as in the odor sensitivity test, take the following measures to avoid cross-contamination between odors and mice.
        1. Mark the water side.
        2. Clean the narrower walls of the cages with two tissue papers sprayed with 70% ethanol, one for each side.
        3. Pile up cages according to the genotype of the mice and store temporarily under a laminar hood.

2. Camera
    1. Mount a camera on a customized tripod with the objective at 58 cm from the bottom of the cage (**Figure 1A**). Fix the position of the Tripod and the cage and delimit with marks to allow for the camera to be centered on the top of the cage.
    2. Record videos at 320 pixels x 240 pixels, 15.08 frames per second as MOV files.

3. Odors
    1. Resuspend the scents, when indicated, in the solvent in which they are soluble.
    2. For the preference test use peanut butter. Resuspend the peanut butter in peanut oil (10% w/v).
    3. For the avoidance test use pure 2-Methylbutyric (2-MB) acid (98%).
    4. For the sensitivity test, use female urine from the same mouse colony and background (C57BL/6).
        1. For convenience collect the urine 1-2 days prior to the olfactory test. Restrain and hold the mouse under the hood with its belly above the cage grid. Under the cage grid place a plastic petri dish to collect the drops of urine.
        2. Collect the urine from each female in a 1.5 ml tube and mix all the urine samples to normalize for variability between animals. Store at -20 °C until use.
        3. On the day of the experiment, thaw the urine and perform 4 dilutions in double distilled water at a dilution factor of 10 (1:10, 1:100; 1:1,000; 1:10,000).

3. Olfactory Testing

Note: In this protocol odors have been deliberately chosen which are perceived as strong attractants (peanut butter and female urine) or strong repellent (2-MB acid)[15]. It is important to carry out the preference and sensitivity tests to pleasant odors prior to the avoidance test to eliminate the possibility of any interference with the olfactory behavior. Nevertheless, for the sake of simplicity, in this paper, preference and avoidance test will be both described under the perception test. Each behavioral session starts with a habituation phase.

1. Habituation Phase
    1. Place the animal in the clean assigned cage and let it explore for 5 min (**Figure 1B**). Since the environment of the experimental cage is familiar to the home cage, this short time is enough to allow for habituation.
    2. If the sensitivity test is completed in one day, perform habituation only once before the application of the highest diluted odor. If the sensitivity test is carried out on different days, on each day a habituation phase on a new clean cage is needed.

2. Perception Test
    1. After habituation, activate the camera and immediately pipette 60 µl of the pleasant scent (peanut butter) and 60 µl of the neutral scent (tap water) onto the opposite walls of the cage at about 10 cm from the bottom (**Figure 1C**).
    2. Let the mouse explore the odors for 2 min (**Figure 1D**). Thereafter, switch off the camera.
    3. At this point, proceed with the next mouse starting from the habituation phase. Perform the avoidance test exactly in the same way by applying 60 µl of the repellent odor (2-MB acid) and 60 µl of water.

3. Sensitivity test
    1. Evaluate the attraction threshold of male mice to increasing concentrations of female urine in the following order: 1:10,000; 1:1,000; 1:100; 1:10 and pure urine.
    2. After habituation, expose each mouse to the highest dilution pipetted by the experimenter as previously described in 3.2.1.
    3. Record the exploratory behavior of urine versus water, within a 2 min time frame on a video camera. After all mice cohorts are tested for the highest dilution (1:10,000), expose to a higher concentration of urine, as indicated above.

4. Post-hoc Data Analysis

Note: All behavioral tests described are processed *post hoc* following the data analysis instructions.

1. Open MOV Files in ImageJ for Windows systems
    1. Install Quick Time for Java using the customized settings from http://www.apple.com/quicktime/download .
    2. Install the Quick Time plugin from the ImageJ website (http://rsb.info.nih.gov/ij/plugins/qt-capture.html).
    3. Import the QTJava.zip (C:\Program Files\QuickTime\QTSystem) into the library extension of ImageJ (.ImageJ\jre\lib\ext).
    4. Copy also the QTJava.zip in the plugins folder and rename it as QTJava.jar.
    5. Install the six scripts attached in the Macros folder (ImageJ\plugins\Macros).
    6. Open ImageJ and compile and run the Quick Time plugin, thereafter close ImageJ.
    7. Reopen ImageJ and open the MOV file using File> import> using Quick Time.

2. Video Adjustment
    1. Once the video file is opened in ImageJ, cut the video in order to obtain a constant 2 min exploration from the time the experimenter has pipetted the odorants into the cage (T0). Identify the frame corresponding to the T0 and remove the previous frames using increments of 1 (ImageJ\Image\Stacks\ToolsSlice remover). Use the same command to delete all the frames exceeding the 2 min exploration.
    2. Make sure that the cage is centered and if necessary use the Image>Transform>Rotate command to align it.

3. Video Processing
    Note: Video processing is fully computer-assisted and uses macros commands accompanying this paper.
    1. In order to restrict the area onto the cage of a 127 pixels x 218 pixels size run the Step 1 macro from the Plugin>Macros>Run command. Move the fixed rectangle over the cage (**Figure 2,** Step 1).
    2. Crop the area of the cage on the region of interest (ROI) using the Step 2 macro (**Figure 2,** Step 2).
    3. Use the Step 3 macro to extract the mouse image from the background by assigning a threshold signal, despeckling and filtering the signal variance. The output values in the Z axis plot indicate the mean grey values, corresponding to the intensity of the mouse shadow moving within the ROI of the “water chamber” during the 2 min exploration. Copy the results into a worksheet named according to the ROI in a spreadsheet file (**Figure 2,** Step 3).
    4. Use the Step 4 macro to extract the mean grey values of the mouse in the ROI “odor chamber”. Copy the results into a worksheet named according to the ROI in the same spreadsheet file as in 4.3.3 (**Figure 2,** Step 4).
    5. In order to further restrict the analysis of the mouse movement in the ROI “water perimeter” use the Step 5 macro. Copy the result onto the worksheet named according to the ROI in the same spreadsheet file as in 4.3.3 (**Figure 2,** Step 5).
    6. To restrict the analysis of the mouse movement in the ROI “odor perimeter” use the Step 6 macro. Copy the result onto the worksheet named according to the ROI spreadsheet file as in 4.3.3 (**Figure 2,** Step 6).
    7. Process all videos and check for consistency in the number of frames per animals. Here, record all animals for 1,810 frames corresponding to a 2 min exploration session.
    8. For each animal and for each ROI sort frames with mean grey values larger than 0. Divide the number of frames by the values corresponding to 1 second and obtain the seconds spent in each ROI.

5. Statistical Analysis

1. For each test verify homogeneity of variance within groups/ genotypes by Bartlett’s test using the formula available on http://www.real-statistics.com/one-way-analysis-of-variance-anova/homogeneity-variances/ .
2. In the attraction and avoidance test, carry out comparisons between the times spent with water versus odor within one group using a non-directional Student’s t-test assuming equal or unequal variances depending on the results of the Bartlett’s test. Compare the times spent with the odors subtracted by the time spent with water between the genotypes by one-way ANOVA with Bonferroni’s *post-hoc* test.
3. In the sensitivity test analyze the comparisons of the time spent with the odor subtracted from the time spent with water among groups at a specific dilutions of urine by one-way ANOVA with Bonferroni’s *post-hoc* test. Compare the sensitivity among groups to growing odor concentrations by 2-Way ANOVA with repetitions with Bonferroni’s *post-hoc* test.
4. Interaction between genotypes and treatments in the attraction and avoidance test are investigated by 2-way ANOVA with Bonferroni’s *post-hoc* test.

Representative Results

The perception test measures the attraction to peanut butter and avoidance to 2-MB acid. Three groups of mice are tested and the time spent in the “odor perimeter” are quantified as compared to water. In the preference test, the control group A displays significant preference to the odor as compared to water ($t_8 = 2.52$, $p < 0.05$). On the other hand, group B does not show any significant attraction to peanut butter and spends more time with water ($t_6 = 3.22$, $p < 0.05$). Thus, it behaves differently from control group A ($F_{1,7} = 26.39$, $p < 0.005$). In addition, group C shows no discrimination and spends about the same time with water and peanut butter ($t_8 = 0.78$, $p = 0.45$). On the whole, the three groups behave differently ($F_{2,9} = 19.83$, $p < 0.005$) and there is a significant interaction between the genotype and treatment (peanut butter and water) ($F_{2,1} = 4.90$, $p < 0.005$) (**Figure 3A**).

In response to 2-MB acid the control group displays an avoidance reflex and as a result spends more time with water ($t_8 = 2.67$, $p < 0.05$). Similarly, group B shows a pronounced avoidance reflex to 2-MB acid ($t_6 = 3.71$, $p < 0.01$). On the other hand, group C does not discriminate between the two odors and spends comparable times with 2-MB acid and water ($t_8 = 2.2$, $p = 0.6$) (**Figure 3B**). On the whole, comparing the

avoidance response the three groups do not display a significant different behavior ($F_{2, 9}$ = 0.76, p = 0.49) as a result there is no interaction between treatment and genotype ($F_{1, 2}$ = 0.52, p = 0.63).

In the olfactory sensitivity test to female urine, the curve displays the preference to urine at different concentrations versus water (Preference index= time spent with urine subtracted by the time spent with water). In this test, we observe that control group A has an attraction threshold to urine at a dilution of 1:1,000 and displays increasing attraction to urine with rising concentrations. Group B and C display a 100-fold higher threshold to attraction (1:10) as compared to group A ($F_{2,9}$ = 4.78, p <0.05). Group B and C display comparable sensitivity curves ($F_{1,19}$ = 0.36, p = 0.55). Comparing the sensitivity among groups, it appears that group A has higher sensitivity to female urine as compared to group B and C ($F_{2,19}$ = 7.12, p <0.01) (**Figure 4**).

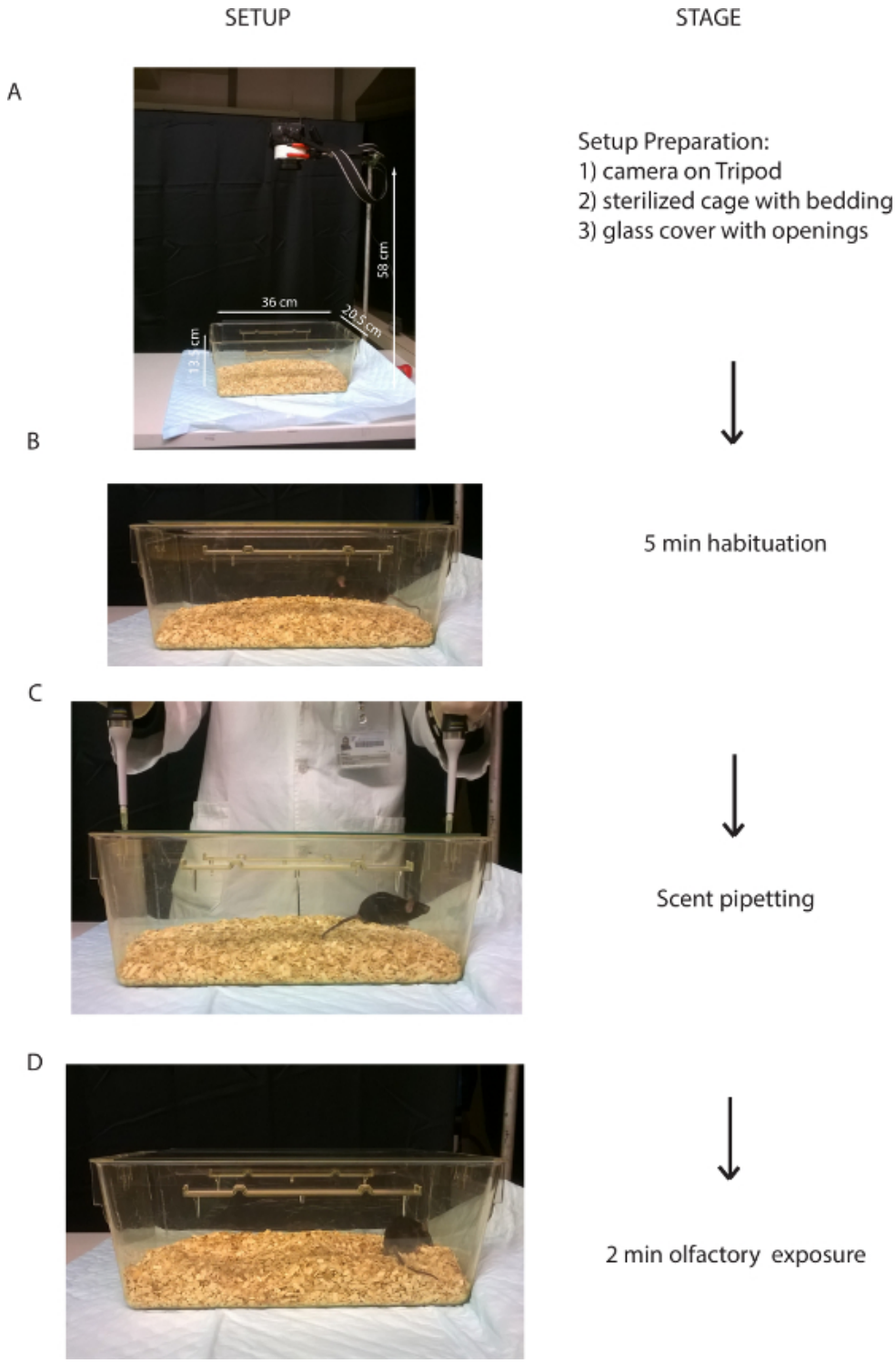
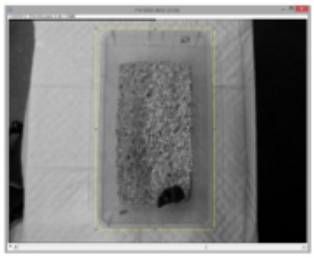
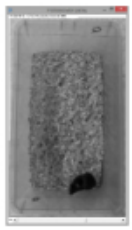
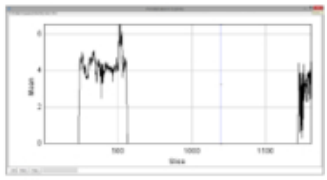


Figure 1: Representation of the setup used to perform the olfactory tests. (**A**) Camera above the cage. (**B**) Mice are placed in a cage for a 5 minutes habituation period. (**C**) The odorants are pipetted on the wall of the cage. (**D**) The exploratory activity of an odorant versus water is tested in a 2 min window.

MACRO	ROI	OUTPUT
1. makeRectangle(0, 0, 127, 216)		N/A
2. run("Crop")		N/A

3. setAutoThreshold();
//run("Threshold...");
setThreshold(0, 30);
run("Threshold", "thresholded remaining black stack");
run("Despeckle", "stack");
run("Variance...", "radius=1 stack");
makeRectangle(0, 0, 127, 108);
run("Plot Z-axis Profile");
		

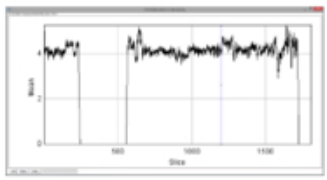
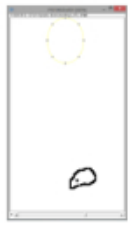
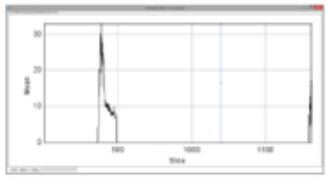
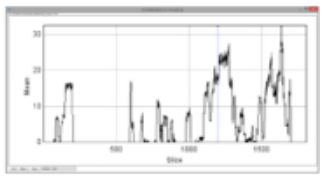
4. makeRectangle(0, 108, 127, 108); run("Plot Z-axis Profile");		
5. makeOval(41, 0, 41, 51); run("Plot Z-axis Profile");		
6. makeOval(41, 164, 41, 51); run("Plot Z-axis Profile");		

Figure 2: Workflow of computer-assisted video processing using macros commands in ImageJ. The example refers to a mouse from group A exposed to urine at a 1:10 dilution. Please click here to view a larger version of this figure.

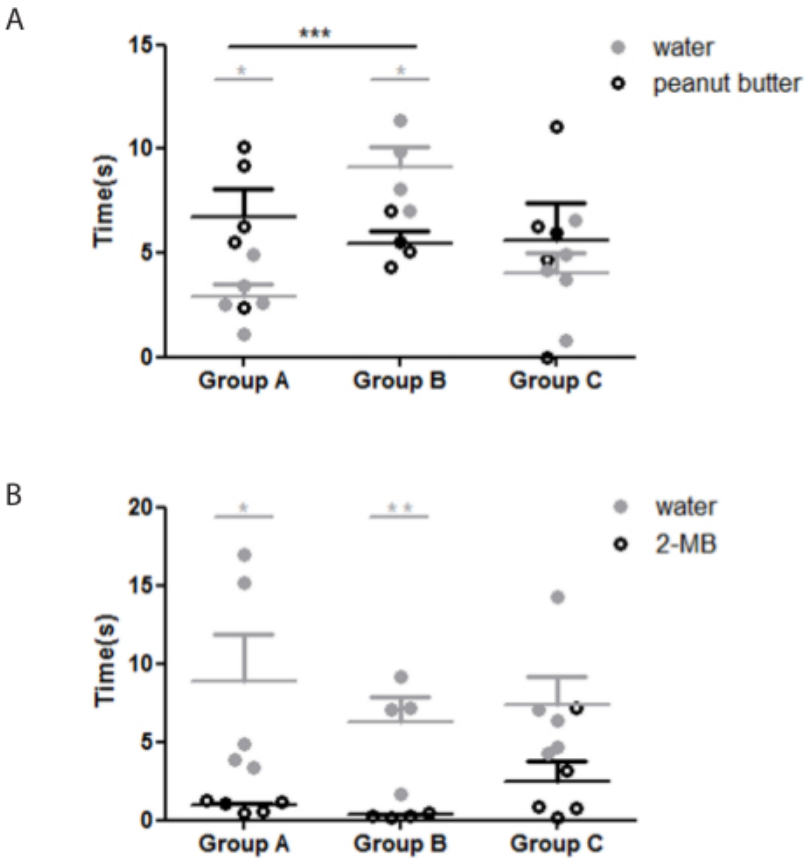


Figure 3: Representative results of olfactory preference and avoidance tests. The mice of the three groups (n = 5 for group A, n = 4 for group B and n = 5 for group C) have been exposed to (**A**) peanut butter and (**B**) 2-MB acid for a 2 min exploration session. The total time exploring the odor (black circles) versus water (grey circles) is represented. Significant differences in olfactory behavior among groups are indicated by black horizontal bars and asterisks. Significant differences in sniffing times between the odor and water within groups are shown by grey horizontal bars and asterisks. *P <0.05, **P <0.01, ***P <0.01 (grey horizontal bars, Student's t-test; black horizontal bar, one-way ANOVA). Error bars are standard errors of the mean (SEM).

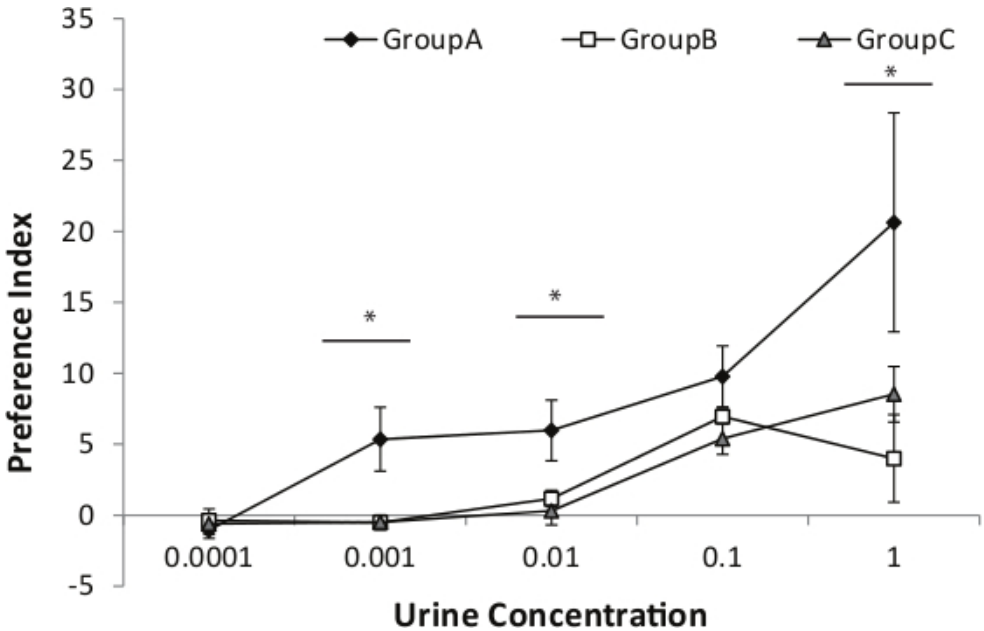


Figure 4: Representative results of sensitivity tests to increasing concentrations of female urine. The preference index curve, given by the exploration time with urine at different concentrations subtracted by the time spent with water, shows that group A (n = 5) has the highest sensitivity to urine as compared to group B (n = 4) and C (n = 5). *P <0.05 (black horizontal bars, one-way ANOVA). Error bars are SEM.

Discussion

The tests proposed in this protocol allow to evaluate different aspects of innate olfactory behavior in mice: perception to odors, discrimination between odors versus water and sensitivity to odors. This protocol can be applied to any odor according to the preference and avoidance scale previously shown[15]. Since the protocol is based on exploratory activity it is important that mice do not display any motor impairment or anxiety which may affect their movement and interfere with olfactory exploration. The tests described are intended for adult male mice however they can be adapted to investigate olfaction also in adult females or aged mice.

Before commencing such a study investigating olfaction in mice it is important to pay attention to the following aspects: 1) perform each test at an interval of at least 3 days. Avoidance should be tested as last to minimize interference of olfactory memory[20]; 2) perform the experiments at the same time of the day, preferably in the late afternoon, when the mice are in their active cycle[21] and use a dimmed source of light. In addition, scheduling the olfactory testing at defined times controls for possible circadian changes in olfactory functions[22]; 3) before starting the avoidance test, which uses repellent odorants, such as acids, bring one cage at the time in the experimental suite and keep the cage under a laminar hood. This step is important to avoid habituation to the odorant and obtain a more homogeneous response in the same group; 4) temporarily separate the mice which have been tested until all mice of the same cage are exposed to the odorant, to minimize odorant contamination; 5) use animals of the same strain, since different strains can behave in a heterogeneous manner when exposed to an odorant[23]; 6) the experimenter should wear a lab coat at all times and change gloves between animals to prevent odor mixing; 7) after pipetting the operator should move slowly away from the cage at a distance of 1.3 meters to prevent any confounding stimulation to the mice during the olfactory exploration; 8) mice displaying mean grey values only in one chamber should be excluded from the study, since mice are expected to explore both chambers to different degrees.

The method described offers several advantages over other protocols: it is extremely simple to set up, uses inexpensive materials, it is of fast completion and takes advantage of open source software, such as ImageJ. In addition, we provide macros that are ready to be installed and which can be custom-used and adapted to any arena and more than 2 odor perimeters. It has to be noted that only the time spent in the assigned odor perimeter is a measure of olfactory activity. Whereas the time spent in each chamber gives a readout of the exploratory activity of the mouse and is only a rough estimation of the olfactory behavior. As with other methods, statistical power can be gained by increasing the number of animals per group.

As compared to the olfactory testing using olfactometers, which can control automatically for vapor pressure and delivery time[12–14], the proposed protocol is less controlled. Nevertheless, all the odors are applied in equal volumes, at defined distance and for the same time window. Thus, keeping these variables constant, in this testing an olfactometer is not required. There is another potential limitation to this protocol consisting in the time required for the adjustment and cutting of each video to obtain a fixed number of frames. Nevertheless, the same computer-assisted analysis can be used also on more sophisticated setups with odor ports delivering the odor at specific times. In this case, the video cutting could be automatically set.

As compared to other protocols using cotton pads impregnated with odor to test attraction and avoidance, the present protocol provides an additional information about the olfactory discrimination between a novel odor and a neutral odor (water)[15,16] in one single experimental session. Moreover, the protocol does not require experimental blinding and can be entirely conducted by a single experimenter using the unbiased computer-assisted analysis.

These simple tests can be used to monitor the progression of neural deficits in Alzheimer's or Parkinson's disease mouse models and to investigate mechanisms of olfactory transmission.

Disclosures

There is no conflict of interest.

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