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Odor preferences shape discrimination learning in rats

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Abstract

Forced-choice discrimination is a standard behavioral paradigm used to test animals' abilities in learning and memory. In this type of task, a reward association is made between a sensory stimulus and a food or water reward and the frequency of correct choice for the stimulus associated with the reward is measured. We here show that when olfactory sensory stimuli are used, spontaneous preferences for odors can influence speed of acquisition in a forced-choice discrimination task. We first show that among a battery of 53 odorants, some odorants elicit longer bouts of spontaneous investigation than others. We confirm that these odor preferences are robust and reliable by measuring relative spontaneous investigation times for pairs of simultaneously presented odorants. Finally, we show that performance on a forced-choice olfactory discrimination depends on relative spontaneous preferences between the rewarded and unrewarded odorants. Namely, rats acquire novel forced-choice odor discrimination problems significantly faster if the preferred odorant, as assessed by relative spontaneous investigation time, is associated with the reward. These results indicate that even subtle differences in the tendency for an animal to approach and investigate one odorant over another can lead to substantial biases in basic learning and memory tasks.

Keywords

Olfactory; forced-choice; preference; behavior; learning

Introduction

One of the standard experimental paradigms for studying olfactory learning and memory is the forced-choice odor discrimination task. In such a task, a reward association is made with one of two odorants and the frequency of correct choice for the rewarded odorant is recorded. This type of task allows an investigator to assess both rate of acquisition i.e., how fast a test subject learns a new discrimination, as well as recall i.e. the test subject's ability to perform the task at a subsequent time point. Typically, forced-choice odor discrimination tasks are executed using monomolecular odorants, such as aliphatic aldehydes, acids, butyrates (Beshel et al., 2007; Cleland et al., 2002) and binary mixtures thereof (Doucette et al., 2007; Uchida and Mainen, 2003). An implicit assumption in most forced-choice

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discrimination tasks is that the task is symmetric with respect to which odorant is rewarded or unrewarded.

Numerous factors can influence a rodent's performance in a forced-choice odor discrimination task. Despite the fact that investigators typically control for stimulus-related factors such as perceptual similarity and intensity of the odorant stimuli (Beshel et al., 2007; Cleland et al., 2002; Doucette et al., 2007; Mandairon et al., 2006), an inherent confound in interpreting results is that relative odor preferences may have an influence on task performance. In particular, if stimulus selection on the early trials is based on relative odor preference, then task performance during these trials is not random but instead depends on whether the preferred odorant is rewarded.

The purpose of the present study was to determine if relative odor preferences among commonly used experimental odorants can influence rats' behavior in a forced-choice odor discrimination task. The results indicate that significant differences in spontaneous investigation can be measured within a battery of 53 commonly used monomolecular odorants and that these differences significantly affect the speed and accuracy of rats' performance in a forced-choice odor discrimination task.

Material and Methods

Subjects

Nine adult (15–25 months) male Long-Evans rats (Charles River Laboratory; Wilmington, MA), weighing 400–450 grams, were used in all experiments. Rats were individually housed and provided unlimited access to water but were maintained on a restricted food diet in order to maintain body weight at 85–90% of *ad libitum* body weight. Rats were kept on a reverse 12 hour light-dark cycle (lights on at 2100), with all experiments taking place during the dark phase of their light cycle (1000–1600). All protocols and procedures were approved by the Cornell Institutional Animal Care and Use Committee.

Odorants

Odor stimuli comprised a battery 53 commonly used odorants (obtained from Sigma-Aldrich, Natick, MA except 2,4,5-trimethylthiazoline, obtained from Contech, Victoria, Canada) listed in Table 1. Odorants were diluted in mineral oil as described in Cleland et al. (2002) so as to theoretically emit a vapor-phase partial pressure of 1 Pa (Experiments 1 and 2) or 0.01 Pa (Experiment 3). Experiment 3 used lower concentrations to render the task more difficult to allow us to observe more subtle effects (Wei et al., 2006).

Experiment 1: Spontaneous odor preferences

Spontaneous preferences for the panel of 53 odorants listed in Table 1 were determined by measuring the amount of time rats spent investigating each odorant when presented in isolation. All experimental testing took place under red light in a standard laboratory cage (46 × 25 × 22 cm) fitted with a slotted metal top (Figure 1A). Rats were habituated to the experimental chamber for one day before starting the experiment. During testing, odorants were introduced by applying 60 μL of odorant onto a piece of filter paper (Whatman #1) in a

weighing dish. The weighing dish was then inverted and placed on top of the cage, which started the timer for the trial. The duration of active investigation, defined as time periods in which the animal's nose was within 1 cm of the weighing dish, was measured during a 120 second trial. The trial ended when the weighing dish was removed from the cage top. One experimenter prepared odors and controlled the start and stop of each trial, while a second experimenter, blind to the identity of the odorant, timed the active investigation. For each of the nine test sessions, rats completed one trial of mineral oil and six trials with unique odorants (drawn from Table 1), presented at five minute intervals. The order in which odorants appeared across testing days, as well as the position of the mineral oil trial within each testing day, was randomized across rats.

To analyze the data, we first normalized investigation times for each rat individually by dividing the investigation for each specific odorant by the average investigation time of that rat on the day that odor appeared. Analysis of variance with posthoc tests (Fisher LSD) was performed on the normalized data.

Experiment 2: Pairwise comparisons

To validate the results from Experiment 1, we chose two odorants that were investigated significantly more than mineral oil (*High*), two that were investigated significantly less than mineral oil (*Low*), and two that were not investigated significantly differently from mineral oil (*Neutral*) for pair-wise comparisons. In this experiment each rat was presented with a pair of odorants at the same time and investigation of each odorant was recorded. Odorants were presented as described for Experiment 1, except that the two weighing dishes were placed at opposite ends of the cage (Figure 2A). Separate experimenters timed the investigation of each odorant. Each rat was tested on all possible across-category pairwise combinations of odorants with the exception of pairing *neutral* odorants against each other (Table 2). Order of the combinations was randomized for each rat, with the constraint that odorants appeared at most once per test session. Each rat ran three trials per day. Data were analyzed by comparing relative investigation times ($\mathrm{relative\_time}_{A,B}=\frac{t_A}{(t_A+t_B)}-0.5,$ where t_A and t_B are the raw investigation times for the two odors in a pair) between conditions followed by multiple comparisons (paired t-tests).

Experiment 3: Forced choice discrimination

To test whether spontaneous preferences among odorants shape odor discrimination learning, we performed a two-alternative forced-choice odor discrimination task. The task took place in a large Plexiglas chamber (50.5 × 40.5 × 30.5 cm), fitted with a vertical divider (Figure 3A). Over the course of several days, rats were habituated to the experimental chamber and shaped to retrieve 45mg sucrose pellets (Bio-Serv, Frenchtown, NJ) from a small depression (1cm diameter, 2 cm depth) in the center of a white sponge (9 cm diameter, 3 cm depth) inside a ceramic dish (9 cm diameter, 4.5 cm depth). Subsequently, each rat underwent training for five days to learn the forced-choice odor task. The odor sets and concentrations for training and testing are listed in Table 3. Training served the purpose of allowing the rats to learn the forced-choice discrimination using pairs of odorants not in the set of experimental odorants. During each training and testing session, rats performed a

novel two-odor discrimination task, with one odorant assigned as the rewarded stimulus. Odorants were presented to the rat by placing 60 μL of odorant on the edge of a sponge; the rewarded sponge contained one 45 mg sucrose pellet. At the start of each trial, the two sponges were placed in the chamber on the opposite side of the divider as the rat. The orientation of the two sponges was randomized so that location was not a cue to reward. The chamber divider was lifted, allowing the rat to approach and investigate the sponges. Stimulus selection occurred when the rat put its nose into the depression of either sponge. Self-correction was not allowed; immediately after the rat selected a sponge it was placed back on the other side of the chamber and the divider was re-inserted. Correct selection was followed by a 20 second inter-trial interval; incorrect selection was penalized by an additional 25 second time-out period. Rats completed 38 trials per session. Odorants were paired to test how rewarding a *High* versus a *Low* or *Neutral* odorant affects the acquisition of and asymptotic performance on the discrimination task. Each rat was tested on one combination each of *High*-rewarded/*Low*-unrewarded, *Low*-rewarded/*High*-unrewarded and *Neutral*-rewarded/*Neutral*-unrewarded. No rat was tested on any given odorant twice and the order of testing was randomized among rats.

Data were analyzed as number of correct trials over all 38 trials, number of correct trials during five-trial blocks or the number of trials needed to reach criterion performance (at least 8 correct trials on two consecutive blocks of ten trials with 50% overlap). Analysis of variance with posthoc tests (Fisher LSD) was used to determine whether there were differences among the odor sets.

After completing all experimental sessions, each rat performed a control session with both sponges scented with the same odorant [(+)-limonene] to ensure that they could not solve the task by using odor or visual cues from the sucrose reward.

Results

Experiment 1: Spontaneous odor preferences

We determined the spontaneous investigation behavior for rats (n=9) in response to each of the 53 odorants listed in Table 1. Odorants appear in Table 1 in order from smallest to largest spontaneous investigation time. Rats show significant variation in investigation times across odorants [$F(52, 477) = 1.954$; $p < 0.001$]. As a control, we used crushed rat food pellets (Teklad LM-485, Harlan Laboratories) in mineral oil; these were investigated significantly more than all other odors ($p < 0.001$ for all combinations, data not shown), showing that the principle of the experiment worked. Figure 1B depicts the individual normalized odor investigation times relative to mineral oil (t_{MO}). The odorants fall broadly into three categories: *Low* odorants had investigation times at least one standard deviation below t_{MO}, *High* odorants had investigation times at least one standard deviation above t_{MO}, and *Neutral* odorants had investigation times within ± one standard deviation of t_{MO}. All odorants were presented at the same theoretical vapor-phase partial pressure; furthermore, the Pearson correlation coefficient between liquid-phase concentration and investigation time is not significant (r=0.096), suggesting that preferences were largely based on odor identity as opposed to intensity.

Experiment 2: Pairwise comparisons

From the panel of odorants used in Experiment 1, we picked six odorants for further investigation, two each from the *Low*, *Neutral*, and *High* categories (Table 2). We then presented rats with pairs of these odorants simultaneously and recorded investigation times. The raw investigation times for each odor pair were converted to relative investigation times (see Methods). Figure 2B displays the average relative investigation times for each type of comparison made (i.e., *High*-*Low* etc…). Analysis of variance yields a significant effect of odorant combination [$F(13, 126) = 8.402$, $p < 0.001$], suggesting that relative investigation time depends on the type of odors in the odor pair. The results of significance tests comparing the relative investigation time on each individual pair of odorants tested is listed in the right-most column of Table 2. In general, rats investigated *High* odorants more than *Low* odorants when paired together, *High* odorants more than *Neutral* odorants when paired together and *Neutral* odorants more than *Low* odorants when paired together. On the other hand, two *High* odorants were equally investigated, as were two *Low* odorants, suggesting that rats exhibit similar preferences for odors drawn from the same category. By directly comparing investigation behavior on pairs of odorants, these results confirm the findings of Experiment 1 and further suggest that our group of rats exhibits a robust and reliable pattern of spontaneous preferences among standard odorants.

Experiment 3: Forced choice discrimination

To test whether spontaneous odor preferences can affect odor discrimination, we performed a forced-choice discrimination task in which either a *High*, *Low*, or *Neutral* odorant was rewarded and paired with a second odorant that was unrewarded. In this experiment, odorants were diluted 10-fold as compared to Experiments 1 and 2 in order to slow down acquisition so that more subtle effects could be observed (Wei et al., 2006). Figure 3B shows the average performance across all trials for each of the three experimental conditions (see Table 3). Overall, we found a significant effect of odor-reward combination on total number of correct trials [($F(2, 24) = 8.921$; $p = 0.001$], with performance on *High*-rewarded tasks being significantly higher than that observed on *Low*-rewarded and *Neutral*-rewarded tasks. On the other hand, overall performance of rats in *Low*-rewarded tasks is not significantly different from that observed in *Neutral*-rewarded tasks.

Differences in average performance across the duration of a discrimination session could be due to differences in the rate of learning and/or differences in asymptotic performance. We found a significant effect of odor-reward combination on learning rate, defined as the number of trials to reach criterion performance [$F(2, 24) = 3.810$; $p = 0.037$]. In particular, rats required significantly fewer trials to reach criterion performance on *High*-rewarded tasks as compared to *Low*-rewarded tasks (Figure 3C). Figure 3D shows the acquisition curves for *High*-rewarded and *Low*-rewarded tasks over blocks of five trials. While differences in performance are evident during the early trials in the task, asymptotic performance (on the last block of trials) is not significantly different between the groups. Thus, despite differences in acquisition speed, rats are ultimately capable of performing each of the discrimination tasks with similar accuracy.

To ensure that rats were not solving the task by detecting visual or odor cues from the sucrose pellet itself, we ran a control experiment in which both the rewarded and unrewarded sponges were scented with the same odorant [(+)-limonene]. Performance on this task averaged 51.8% and was not significantly different from the expected value of 50%, indicating that rats were not able to solve the task simply by detecting the sucrose pellet.

Discussion

We show that relative preferences for odors commonly used in olfactory learning and memory studies can significantly affect the outcome of such behavioral experiments. Rats exhibit longer investigation times in response to some commonly used odorants, such as eugenol or anisole, whereas they were less likely to approach and investigate other odorants such as isoamyl acetate or acetic acid (Figure 1B). The observed pattern of preferences for odorants that elicited longer and shorter bouts of spontaneous investigation (relative to mineral oil) were equally obvious when rats were given the opportunity to investigate two odorants at the same time (Figure 2B).

We next showed that speed of acquisition in a forced-choice discrimination task depends significantly on the choice of rewarded odorant. When an odorant that elicits a high amount of spontaneous investigation is rewarded, rats require fewer trials to achieve criterion performance than when an odorant that elicits a low amount of spontaneous investigation is rewarded (Fig. 3B). We hypothesize that the spontaneous preference for the *High* odorant biases the rat to preferentially select that odorant during the early trials of the task, when the probability of stimulus selection is otherwise theoretically assumed to be equal for the two odorants. In other words, if the *High* odorant is rewarded, rats are more likely to choose it during the initial trials and may acquire the discrimination faster. This idea is supported by comparison of the acquisition curves (Fig. 3D), which show more correct choices during the first five trials in the *High*-rewarded case than in the *Low*-rewarded case. Thus, a small bias toward one odor in the pair can significantly affect learning rate. However, for all odor pairs tested, rats reached equivalent levels of asymptotic performance, suggesting that the small biases associated with relative odor preferences are easily overcome when motivated by food reward.

The odor preferences exhibited by an adult animal are presumably shaped both by genetic factors as well as pre- and post-natal experience. We have used an in-bred laboratory rat strain for these experiments; however, we have no knowledge of the rats' odor-related experiences before arrival at our animal facility, and little control over the odor experience when the rats are not in our laboratory. Thus, while it is tempting to speculate that there may be a genetic basis for the observed preferences, we hesitate to draw such conclusions. Moreover, we also intentionally avoid ascribing valence to the odor preferences based on our behavioral analysis. In this study, we quantify "preference" by using a behavioral metric of active odorant investigation. Although longer investigation behavior may be interpreted as a form of attraction (and conversely short or no investigation behavior as a form of repulsion or avoidance), in fact, investigation can more reasonably be interpreted as information acquisition. That is, rats approach and investigate an odor source in order to obtain further information. Interestingly, an odorant previously categorized as a repellent in rodents,

trimethylthiazoline (Endres et al., 2005), was more strongly investigated in our study and ultimately classified among the *Neutral o*dorants. Thus, an odor with a theoretically strong negative hedonic value elicits the opposite behavioral reaction expected. It is possible that, at the concentration tested, the rat was unable to identify the odor and therefore approached it in order to aid identification. Regardless, this observation cautions against attributing valence to our behavioral metric.

In general, our results show that subtle biases in odor preferences can result in significant differences in learning on olfactory discrimination tasks, suggesting that, in addition to factors such as odor similarity and concentration, spontaneous preferences is an important variable to consider in the design of olfactory behavioral studies. It remains to be tested if such relative preferences towards simple stimuli are unique to olfaction or are relevant in other sensory systems as well.

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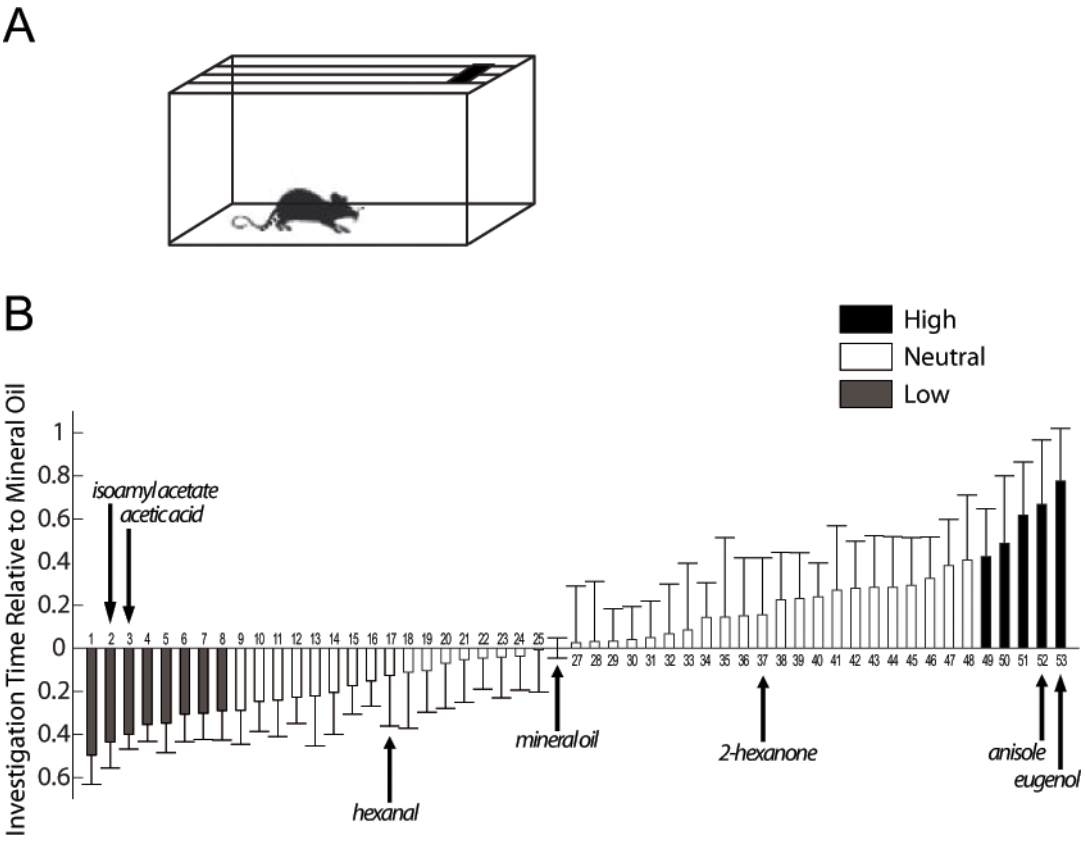

Figure 1.

Spontaneous investigation times vary across a panel of commonly studied odorants. **A,** Schematic of experimental setup. The timer started when the weighing dish (black square) was placed on top of the cage. **B**, Average normalized investigation time (±1 SEM) for each odorant in Table 1. The average investigation time to mineral oil has been subtracted from each data point. We classified odorants into three categories: *High* odorants (black bars) elicited investigation times at least one standard deviation greater than that for mineral oil and *Low* odorants (gray bars) were those with investigation times at least one standard deviation below that for mineral oil. All other odorants were considered to be *Neutral* (white bars).

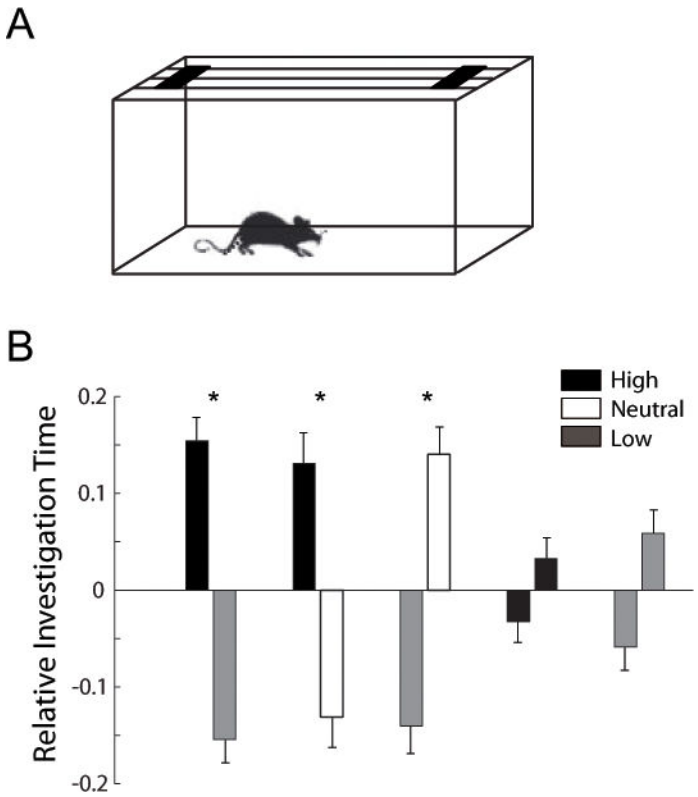

Figure 2.

Pairwise comparisons between odorants with different spontaneous investigation times. **A,** Schematic of experimental setup. The timer started when the weighing dishes (black squares) were placed on the top of the cage. A separate timer was used for each odorant. **B**, Average relative investigation time (±1 SEM) for all pairs of *High-Low*, *High-Neutral*, *Low-Neutral*, *High-High*, and *Low-Low* odorants (see Table 2). *P<0.05.

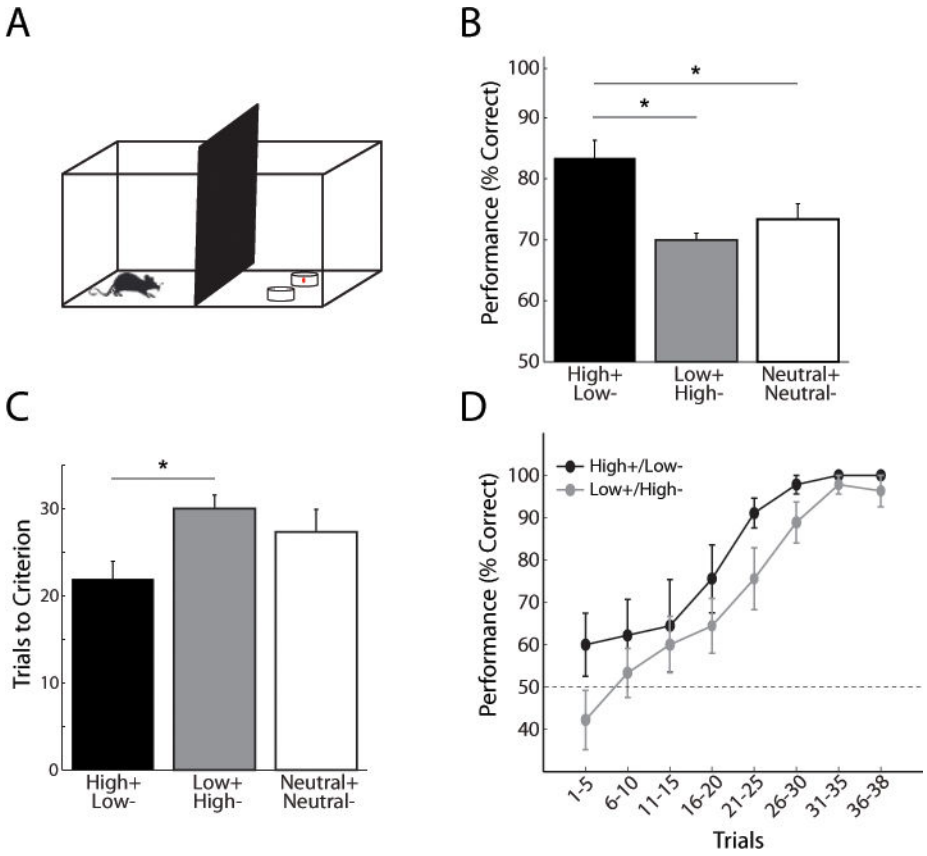

Figure 3.

Differences in spontaneous investigation behavior influence performance in a forced-choice odor discrimination task. **A**, Schematic of experimental setup. **B,** Average performance (+1 SEM) for the three odor-reward combinations used (see Table 3 for specific odor pairs tested). **C,** Average number of trials (+1 SEM) to reach criterion performance for the same three groups. **D**, Average percent correct performance (±1 SEM) for consecutive five-trial bocks for *High*-rewarded (black) and *Low*-rewarded (gray) conditions. In all panels, * denotes a significant difference between groups ($p < 0.05$)

Table 1

List of odorants used in Experiment 1. Odorant ID corresponds to data shown in Figure 1B. All odorants were diluted in mineral oil to theoretically emit a vapor-partial pressure of 1 Pa (Cleland et al., 2002); the resulting percentage volume/volume concentrations are listed. Odorants marked with a * were selected for further study in Experiments 2 and 3.

Odor ID	Name	%v/v
1	hexyl acetate	0.227
2*	isoamyl acetate	0.05
3*	acetic acid	0.0078
4	pentyl butyrate	0.0572
5	heptanoic acid	4.627
6	butyl acetate	0.0219
7	propanol	0.00553
8	hexyl butyrate	1.627
9	propyl butyrate	0.0522
10	butyl hexanoate	1.627
11	methyl butyrate	0.0071
12	1,8-cineole	0.195
13	hexanol	0.255
14	heptanal	0.0707
15	octanal	0.147
16	butyl propionate	0.0604
17*	hexanal	0.0221
18	butyric acid	0.127
19	citronellal	1.658
20	propanoic acid	0.033
21	+terpenin	6.632
22	3-heptanone	0.0646
23	2-heptanone	0.0574
24	n-propyl acetate	0.00627
25	butyl pentanoate	0.572
26	mineral oil	–
27	Pentanol	0.0744
28	ethyl acetate	0.00169
29	2-pentanone	0.0054
30	Butanol	0.0208
31	+limonene	0.204
32	methyl acetate	0.00426
33	n-amyl acetate	0.0723
34	methanol	0.000309

Odor ID	Name	%v/v
35	ethyl butyrate	0.0181
36	1-octanol	2.673
37*	2-hexanone	0.0180
38	pentanoic acid	0.45
39	hexanoic acid	1.488
40	methyl 2-furoate	0.247
41	propanal	0.000485
42	Cyclobutanecarboxylic acid	1.178
43	pentanal	0.00657
44	butanal	0.00185
45	trimethyliazoline	1.00
46	2-methybutyric acid	0.379
47	heptanol	0.838
48	octanoic acid	13.742
49	5-methylfurfural	0.299
50	+carvone	4.716
51	2-furyl methyl ketone	0.259
52*	anisole	0.0515
53*	eugenol	0.0746

Table 2

Odor combinations used in Experiment 2. All odorants were diluted in mineral oil to theoretically emit a vapor-phase partial pressure of 1 Pa (see Table 1). Results of significance tests for differences in relative investigation time for each combination are listed in the rightmost column.

Combinations	Odor 1	Odor 2	ΔRelative Investigation Time
(1) High-Low	eugenol eugenol anisole anisole	isoamyl acetate acetic acid isoamyl acetate acetic acid	*p<0.001* NS *p<0.001* *p<0.001*
(2) High-Neutral	eugenol eugenol anisole anisole	2-hexanone hexanal 2-hexanone Hexanal	*p<0.001* *p<0.001* NS *p<0.01*
(3) Low-Neutral	eugenol eugenol anisole anisole	2-hexanone hexanal 2-hexanone hexanal	p<0.05 *p<0.001* *p<0.01* *p<0.05*
(4) High-High	eugenol	anisole	NS
(5) Low-Low	isoamyl acetate	acetic acid	NS

Table 3

Odor sets used for Experiment 3. All rats completed one discrimination session using each of the five training odor sets (in the order listed), followed by one discrimination problem from each of the three test categories, in random order. All odorants were diluted in mineral oil to theoretically emit a vapor-phase partial pressure of 0.01 Pa, unless otherwise indicated.

	Rewarded Odor	**Unrewarded Odor**
Training Odor Sets:	pentyl butyrate (1 Pa) 5,methyl-furfural (0.1 Pa) citronellal furfural proprionate propanol	heptanal (1 Pa) hexanol (0.1 Pa) butyl hexanoate 3-heptanone ethyl pentanoate
Test Condition 1: *High* rewarded/*Low* unrewarded	eugenol eugenol anisole anisole	isoamyl acetate acetic acid isoamyl acetate acetic acid
Test Condition 2: *Low* rewarded/*High* unrewarded	isoamyl acetate acetic acid isoamyl acetate acetic acid	eugenol eugenol anisole anisole
Test Condition 3: *Neutral* rewarded/*Neutral* unrewarded	hexanal 2-hexanone	2-hexanone hexanal