

Original Article

# Olfactory Detection Thresholds for Primary Aliphatic Alcohols in Mice

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## Abstract

Probing the neural mechanisms that underlie each sensory system requires the presentation of perceptually appropriate stimulus concentrations. This is particularly relevant in the olfactory system as additional odorant receptors typically respond with increasing stimulus concentrations. Thus, perceptual measures of olfactory sensitivity provide an important guide for functional experiments. This study focuses on aliphatic alcohols because they are commonly used to survey neural activity in a variety of olfactory regions, probe the behavioral limits of odor discrimination, and assess odor-structure activity relationships in mice. However, despite their frequent use, a systematic study of the relative sensitivity of these odorants in mice is not available. Thus, we assayed the ability of C57BL/6J mice to detect a homologous series of primary aliphatic alcohols (1-propanol to 1-heptanol) using a head-fixed Go/No-Go operant conditioning assay combined with highly reproducible stimulus delivery. To aid in the accessibility of our data, we report the animal's threshold to each odorant according to the 1) ideal gas condition, 2) nonideal gas condition (factoring in the activity of the odorant in the solvent), and 3) the liquid dilution of the odorant in the olfactometer. Of the odorants tested, mice were most sensitive to 1-hexanol and least sensitive to 1-butanol. These updated measures of murine sensitivity will hopefully guide experimenters in choosing appropriate stimulus concentrations for experiments using these odorants.

**Key words:** behavior, olfaction, psychophysics

## Introduction

To uncover how stimuli are encoded within the brain, it is important to understand the range, sensitivity, and limitations of the sensory system. Perceptual measures of detection threshold provide a mechanism to compare sensitivity across species and gauge appropriate stimulus concentrations to probe sensory coding. In the olfactory system, this information is particularly valuable as each receptor frequently lacks one-to-one specificity with an odorant as even monomolecular odorants can activate multiple receptors in a concentration-dependent manner (Malnic et al. 1999; Kauer and White 2001). Thus, experiments designed to probe odor coding demand the use of adequate stimulus concentrations; however, in most cases, this information is severely lacking.

Primary aliphatic alcohols are commonly used to survey neural activity in a variety of olfactory regions, probe the behavioral limits of odor discrimination, and assess odor-structure activity relationships in mice (e.g., Sato et al. 1994; Malnic et al. 1999; Inaki et al. 2002; Laska et al. 2008; Johnson et al. 2009; Xu and Wilson 2012; Iurilli and Datta 2017; Liu et al. 2018). The stimulus concentrations used in these studies vary widely as no systematic survey of the relative sensitivity to these odorants exists in this species. A systematic survey of aliphatic alcohol sensitivity is available for another rodent species, the rat (Moulton and Eayrs 1960). However, the unusual method of stimulus delivery (small nylon capsules attached to a water bottle) would seemingly lessen the applicability of these sensitivity measures for functional studies employing more conventional methods of odor delivery. Behavioral thresholds for individual aliphatic alcohols (using

flow-dilution olfactometers) are available for both rats and mice but differ in their behavioral method, the manner of odor delivery, the solvent used, and even the definition of threshold (mice: Deiss and Baudoin 1997; Youngentob and Margolis 1999; Larson et al. 2003; Pho et al. 2005; Smith et al. 2008; Lotvedt et al. 2012; rats: Laing 1975; Youngentob et al. 1997; Yoder et al. 2017). Thus, depending on the study, sensitivity estimates for an odorant can differ by many orders of magnitude. One potential consequence of this variability and lack of a systematic analysis is that functional studies may be employing higher than ideal odorant concentrations.

Panels of structurally similar odorants are typically presented at equivalent concentrations to map odor-evoked responses throughout the olfactory system (e.g., Uchida et al. 2000; Takahasi et al. 2004). Thus, large variations in behavioral sensitivity toward these odorants could potentially obfuscate the fundamental principles of odor coding. Interestingly, behavioral sensitivity toward aliphatic alcohols appears to be negatively correlated with the carbon chain length in multiple species, including the rat (Moulton and Eayrs 1960), several nonhuman primates (Laska and Seibt 2002; Laska et al. 2006), and humans (Cometto-Muniz and Cain 1990). Although this relationship has not been analyzed in mice, Smith et al. (2008) measured the sensitivity of mice (in the context of a genetic manipulation) to 2 different aliphatic alcohols. In contrast to other species, they found that mice were several orders of magnitude more sensitive to 1-propanol (C3) than 1-heptanol (C7; Smith et al. 2008). Thus, additional work is needed to adequately define the behavioral sensitivity of mice to these commonly used odorants.

The goal of the current study was to determine the olfactory detection thresholds of C57Bl/6J mice (the most commonly used inbred strain) to primary aliphatic alcohols using operant conditioning combined with head fixation and a well-controlled and highly reproducible stimulus delivery system. The results of the current study will hopefully guide experimenters in choosing appropriate concentrations for functional studies using aliphatic alcohols.

## Methods

### Animals

Male and female C57Bl/6J mice (16 M; 16 F) were obtained from Jackson Laboratory and housed in same-sex cages until head-bar surgery. Mice (10–12 weeks old) were anesthetized with isoflurane at a dosage of 2–3% in oxygen, administered buprenorphine (0.1 mg/kg) as an analgesic, and lidocaine (2 mg/kg) as a local anesthetic. Mice were secured in a stereotaxic head holder with nonrupture earbars during the duration of the procedure. A custom titanium head bar (<1 g) and 2 or 3 microscrews were affixed to the skull and secured using dental cement. The ID number of the animal was added to the headbar to ensure correct identification throughout the experiment.

After surgery, mice were individually housed and given at least 3 days to recover. Following recovery, mice were water restricted for at least 2 weeks prior to training in a water-rewarded conditioning paradigm. The daily allotment of water for each mouse was determined according to their body weight. Mice that weighed 85–100% of their initial bodyweight received 1 mL of water, whereas mice that weighed 70–85% of their initial bodyweight received between 1–2 mL of water. All procedures conducted were reviewed and approved by the Florida State University Animal Care and Use Committee.

### Odor stimuli

A set of 5 aliphatic alcohols was used: 1-propanol (CAS# 71-23-8) 1-butanol (CAS# 71-36-3), 1-pentanol (CAS# 71-41-0), 1-hexanol

(CAS# 111-27-3), and 1-heptanol (CAS# 111-70-6). All odorants were of the highest available purity (>99%) and obtained from Millipore-Sigma. They were stored under nitrogen and housed in a chemical storage cabinet (Air Science). Odorants were diluted with nanopure water within an odor-free chemical safety cabinet with the use of filtered pipette tips. Water was chosen as the solvent because it is odorless and the activity coefficients of these odorants in water are available (see below). The odorant concentration in the vapor phase under ideal gas conditions was calculated using the vapor pressure for the respective odorant:

$$C \text{ (ppm)} = \frac{P_{\text{vap}}}{P_{\text{atm}}} \quad (10^6)$$

where  $C$  is the concentration in ppm,  $P_{\text{vap}}$  is the vapor pressure of the odorant and  $P_{\text{atm}}$  is the atmospheric pressure in mmHg at 25 °C. The vapor pressures used were: 1-propanol (23.2 mmHg), 1-butanol (7.78 mmHg), 1-pentanol (2.65 mmHg), 1-hexanol (0.881 mmHg), and 1-heptanol (0.299 mmHg). The concentration in ppm was then converted to molarity ( $M^0$ ) through division by the molar volume for each dilution. This number represents the molarity under ideal gas conditions. However, both the volatility and solubility of aliphatic alcohols decreases with carbon chain length. Although we chose C3–C7 alcohols because they represented the best compromise between lower to moderate volatility (allowing stable odor presentations across trials) and their solubility in water, each diluted odorant (in water) deviates from the ideal gas conditions to a different degree (Table 1). Thus, a more accurate odorant concentration in the vapor phase was determined by multiplying the vapor phase molarity in the ideal gas condition ( $M^0$ ) by the activity coefficient ( $\gamma$ ) of each odorant in water for each dilution:

$$M = M^0 \times \gamma$$

To allow our measurements of sensitivity to be compared across the literature, we have included both measures of vapor phase molarity ( $M^0$  and  $M$ ), as well as the liquid dilution of the odorant in the olfactometer (Table 1). However, whenever possible, the nonideal gas odorant concentrations should be used as they are more accurate.

The maximum odorant concentration tested was 1:100 dilution for 1-butanol ( $4.23 \times 10^{-7} M^0$  or  $2.17 \times 10^{-5} M$ ) and 1-pentanol ( $1.44 \times 10^{-7} M^0$  or  $2.81 \times 10^{-5} M$ ), 1:1,000 dilution for 1-propanol ( $1.26 \times 10^{-7} M^0$  or  $1.79 \times 10^{-6} M$ ), and 1:10,000 dilution for 1-hexanol ( $4.79 \times 10^{-10} M^0$  or  $2.64 \times 10^{-7} M$ ) and 1-heptanol ( $1.62 \times 10^{-10} M^0$  or  $3.19 \times 10^{-7} M$ ). These maximum concentrations are well below solubility limits of these odorants in water.

### Stimulus delivery

Odorants were delivered using an 8-channel, flow-dilution olfactometer (Shusterman et al. 2011; Dewan et al. 2018; Figure 1). Disposable 40-mL amber glass vials filled with 5 mL of diluted odorant (or nanopure water) were attached to the olfactometer manifolds and pressurized before the start of the first trial. These manifolds switched between a pressure-balanced empty carrier vial (via normally open solenoid valves) and 7 odorant vials (via normally closed solenoid valves). Nitrogen gas regulated by a 100-mL/min mass flow controller (Alicat Scientific) flows through the selected vial before it is diluted 10 times by the main airflow stream—regulated by a 900 mL/min air mass flow controller (Alicat Scientific). Nitrogen is used in the odorized line to minimize the oxidation of the odorant when the vial is not in use and has no effect on the animal. A dual synchronous 3-way solenoid valve (final valve) connected the olfactometer and a purified air line (~1000 mL/min) to an exhaust line and the odor port. Care was taken to ensure that both lines were impedance matched to limit pressure spikes during odor

delivery. During stimulus delivery, the final valve swapped the flow to the animal from clean air to diluted odorant. The selected vial within the olfactometer is actuated 1.2 s prior to stimulus delivery to allow the odor concentration to reach equilibrium prior to delivery to the animal.

To verify the stability and reproducibility of this method of stimulus delivery, a photo-ionization detector (PID, Aurora Scientific) was used in place of the mouse. A single vial containing a 1:100 dilution of 1-butanol was repeatedly actuated (250 times) with an 8-s intertrial interval. Preliminary tests with other odorants/and concentrations resulted in similar levels of consistency. Please note that the short delay to maximum stimulus concentration (<250 ms) is due to 3 factors: 1) the PID is positioned at the location of the mouse's nose not the odor port, 2) final valve is located outside of the chamber to prevent auditory cues confounding our results, and 3) the ionization rate of the odorant within the PID.

To ensure that our 10-fold liquid dilution steps resulted in a linear decrease in odor concentration, we used the highest and second-highest concentrations tested for all odorants (to maximize the signal to noise ratio). Each concentration was presented 5 times and the PID response was recorded.

### Behavioral assay

Water-restricted mice were trained to report the detection of odor in a Go/No-Go task (Dewan et al. 2018) in a custom-built apparatus (Figure 1). Each cohort of mice initially consisted of 4 males and 4 females (age matched). Cohorts were tested on a maximum of 2 odorants to limit overtraining and minimize the probability that mice were solving the task using nonodor cues (see below). Individual mice were excluded from the experiment if they failed to reach training criterion ( $n = 1$ ) or learned to solve the task using nonodor cues ( $n = 2$ ; see below for specific details). Mice were placed in a custom holder with their nose 1 cm from the odor port. The odor port was mounted on a concave base that housed the lick tube and vacuum connection to remove excess odor. Licks were detected electronically using a lick circuit. Water delivery was controlled by a solenoid valve connected to a small water reservoir. An Arduino-based controller regulated the olfactometer, whereas a different Arduino-based behavioral controller coordinated the trial structure and monitored licks. A Python script (Voyeur software—originally described in Smear et al. 2013) sent trial parameters to the behavioral controller, actuated the olfactometer, and stored all the response data. The animal holder and odor port were mounted on a breadboard inside an 18×18×18" custom-made sound-proof box. A fan mounted on the top of the box removed any residual odor not eliminated by the vacuum connection.

Behavioral training consisted of 2 stages. Stage 1—the mice were trained to receive a water reward (1.5–2  $\mu$ L) if they licked at least once during the 2-s stimulus period (signaled by an LED). Throughout training and testing, a single lick denoted the behavioral response and terminated the stimulus period. The intertrial interval was steadily increased from 1.5 to 8 s over the course of several sessions. During the initial session, mice tended to lick continuously but gradually learned to respond only during the stimulus periods. Mice were exposed to clean air (1000 mL/min) throughout the session either from the purified air line (intertrial intervals) or the olfactometer (stimulus period). Each stage 1 session lasted 900–1200 trials and mice experienced 3–7 sessions before starting stage 2 training. Stage 2—mice were trained in a Go/No-Go odor detection task. A blank olfactometer vial (5 mL of nanopure water) served as the Go stimulus, whereas a vial containing the highest concentration of the target odor served as the No-Go stimulus (see above for concentrations). Correct responses during the 2-s stimulus period were immediately rewarded with water (1.5–2  $\mu$ L) and/or a short intertrial interval (8–10s). Incorrect responses were punished with a longer intertrial interval (13–18s). Intertrial intervals were randomized within these ranges to prevent mice from anticipating trial

start times. Because overmotivation due to increased thirst can mask true sensitivity (Berditchevskaia et al. 2016), the first 10 trials were Go trials and were not included in our analyses (and are not plotted within the figures). Sessions typically lasted 300–800 trials and were terminated after the mice missed 3 Go trials in a row. Behavioral performance was determined by the number of correct responses (hits + correct rejections) divided by the total number of trials (after the initial Go trials). Mice learned this task quickly and usually performed >90% in the second session. Upon reaching criterion (2 sessions >90% correct), mice were subsequently tested in the thresholding assay. Stage 2 training does not include a cheating check (see below), so the maximal behavioral performance is 100% (compared with 85% for the thresholding experiment). Mice that did not reach criterion in a maximum of 4 days were excluded.

To determine behavioral thresholds, mice were only tested at 1 concentration per day. This approach eliminated any masking/adaptation effects resulting from the contamination of the olfactometer by higher concentrations of the target odor. The olfactometer was loaded with 3 blank (Go) vials, 3 diluted odor (No-Go) vials, and a single blank (No-Go) vial. Each vial was replaced daily, and their positions were randomized. The first session used the same concentration as stage 2 training experiment (see above for concentrations), whereas each subsequent session presented the mice with a 10-fold dilution of the odorant. Again, mice typically performed 300–800 trials per session and each session was terminated when the mice missed 3 Go trials in a row. Whereas this approach maximized the length of the session, average behavioral performance stabilized after 150–175 trials (Figure 1d). The total flow rate (but not flow dilution factor) from the olfactometer was fluctuated (970, 980, 990, or 1000 mL/min) on a per-trial basis to limit mice from using slight variations in air pressure (likely associated with small differences in the resistivity of each solenoid/vial combination) to solve the task. The blank No-Go vial (or “cheating check”) served to test whether the mice were using cues other than the presence or absence of the target odor to maximize performance. This blank No-Go vial should be indistinguishable from other blank Go vials unless the animal is using nonodor cues to maximize performance and the associated water reward. Thus, mice are “cheating” at this task if they are able to reject (i.e., not lick) the blank No-Go vial at a frequency higher than the percentage of misses (i.e., not licking during a blank Go vial). If this occurred, the session was excluded from the analysis. If this occurred multiple times over the course of an experiment, the mouse was removed from the experimental group. Because this check is included in our thresholding analysis, the maximum performance a mouse can attain using only odor cues in this experiment is approximately 85% (in contrast to stage 2 training in which the mice can achieve 100% behavioral performance). After the completion of all odor concentrations, the mouse's ability to discriminate between vials using nonodor cues was again tested by loading the olfactometer with only blank vials. These data are included in each figure.

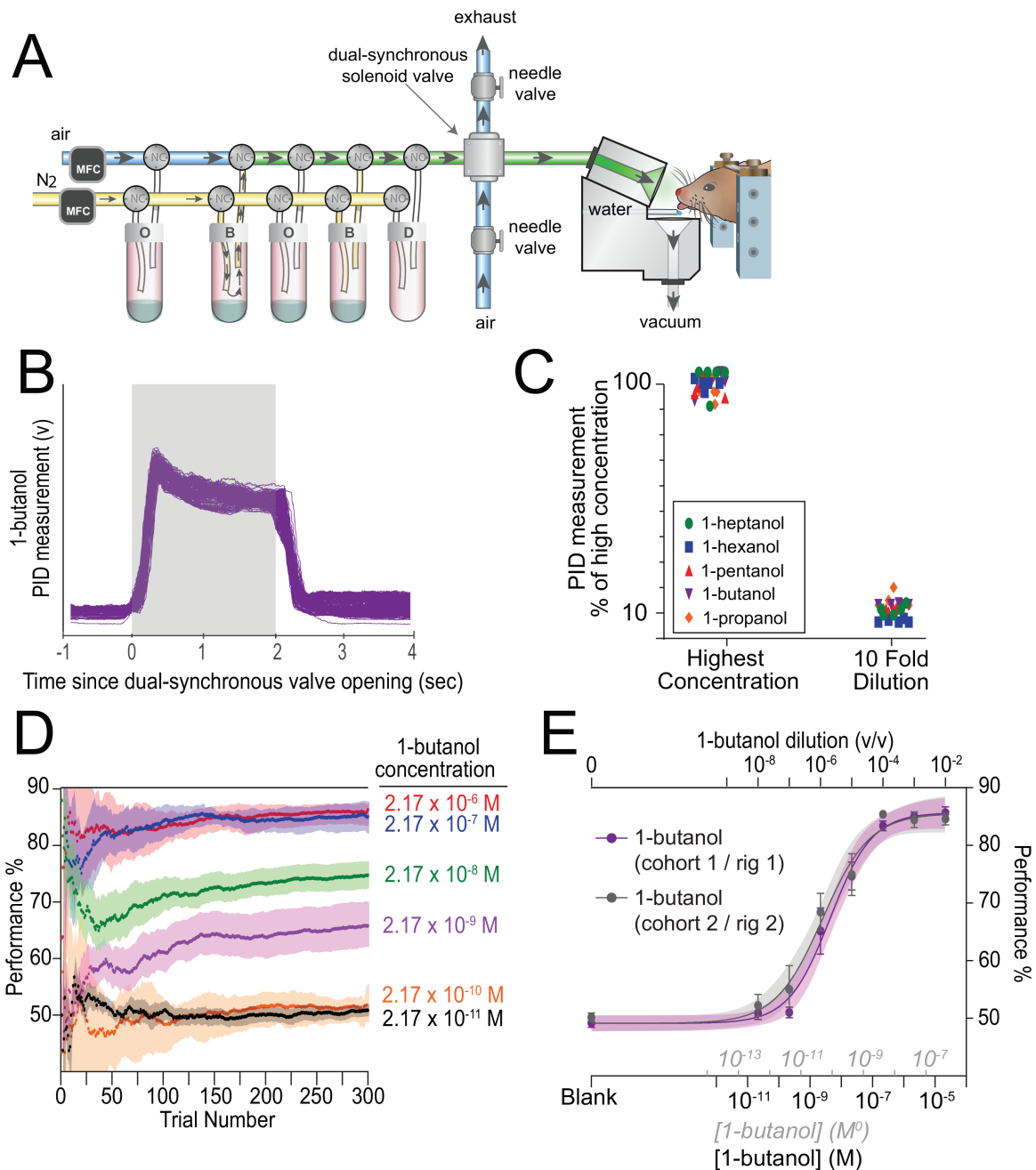
At the end of each day, the olfactometer (including the manifolds and all tubing) were flushed with 70% isopropanol, followed by nanopure water, and dried with pressurized clean air overnight. The vial caps and tubing were also cleaned with isopropanol, followed by nanopure water and dried overnight.

### Data analysis

Behavioral performance data for each odor were fitted with a Hill function.

$$R = R_{\min} + \frac{R_{\max} - R_{\min}}{\left[1 + \left(\frac{C}{C_{1/2}}\right)^n\right]}$$

where  $R$  is the behavioral accuracy,  $C$  is odor concentration,  $C_{1/2}$  is the concentration at half-maximal performance, and  $n$  is the Hill coefficient.



**Figure 1.** To measure behavioral detection thresholds, we used a head-fixed Go/No-Go operant conditioning assay combined with well-controlled and highly reproducible stimulus delivery. **(A)** Odors are delivered using an 8-channel flow-dilution olfactometer that switches between a pressure-balanced dummy (D) vial (via normally open valves, NO) and either odor (O) or water (B) vials (via normally closed valves). Odorized air is directed to exhaust to allow the stimulus to reach equilibrium prior to stimulus delivery. During stimulus application, a dual-synchronous solenoid valve redirects pressure-balanced, odorized air from exhaust to the animal. At the conclusion of the trial, the dual-synchronous solenoid valve returns the pressure-balanced clean air to the animal. **(B)** PID traces of 250 stimulus presentations of 1-butanol. Shaded area signifies 2-s stimulus period. **(C)** 10-fold liquid dilution steps appear to exhibit the predicted decline in PID response. **(D)** Average cumulative behavioral performance across 300 trials for several concentrations of 1-butanol. Initial Go trials are not included. Line signifies mean with shaded standard error (SE). Final behavioral performance for each concentration is plotted in the next panel (cohort 1/rig 1). **(E)** Our experimental approach did not differ across mouse cohorts or different behavioral setups/olfactometers. Data were fitted using a hill function. Maximal behavioral performance for each odor concentration is limited to ~85% (see Methods). Plots show mean  $\pm$  SE with shaded 95% CI.

We defined threshold in the standard psychophysical manner as the concentration at which mice discriminate the odor from blank with 50% accuracy ( $C_{1/2}$ ), typically represented by the inflection point of the psychometric curve (for a more detailed description, see Harvey 1986). The  $C_{1/2}$  values were compared between odorants using a sum-of-squares *F*-test (Prism Graphpad).

## Results

To measure behavioral detections thresholds, we used a head-fixed Go/No-Go operant conditioning assay combined with well-controlled and highly reproducible stimulus delivery (Figure 1a). Using this setup, repeated actuation of a single odorant vial resulted

in consistent odor kinetics that had <250 ms delay to peak concentration at the mouse's nose (Figure 1b). Importantly, mice responded to the stable portion of the odor presentation and not the rapid increase (Supplementary Figure S1). Across all 1-butanol concentrations, the average response time for a correct Go response was  $545 \pm 133$  ms (8619 trials) and  $561 \pm 184$  ms (3606 trials) for an incorrect No-Go response (min: 389 ms, max: 1918 ms for all responses). Response times were not correlated with odor concentration (Go:  $r_s = -0.237$ ;  $P = 0.608$ , No-Go:  $r_s = 0.675$ ;  $P = 0.097$ , Spearman correlation). The 10-fold liquid dilution steps used in the experiment exhibit the predicted linear decline in the PID response (Figure 1c). Although sessions typically lasted more than 300 trials (see Methods for session termination criterion), behavioral performance could be accurately predicted after 150–175 trials (Figure 1d). Our approach is not only relatively consistent among individuals (see below) but even across cohorts of animals. The sensitivity to 1-butanol across 2 different cohorts of mice tested in different behavioral chambers (connected to different olfactometers) was similar ( $P = 0.29$ ,  $F = 1.13$ , sum of squares test; Figure 1d).

The average vapor phase detection threshold for 1-propanol was  $1.3 \times 10^{-10}$  M (95% CI:  $0.8\text{--}1.8 \times 10^{-10}$  M) or 0.003 ppm (Figure 2a,f; Table 1). These thresholds were equivalent to a  $7.1 \times 10^{-8}$  (95% CI:  $4.7\text{--}10.4 \times 10^{-8}$ ) liquid dilution of 1-propanol in water (v/v) with a 10% air dilution of the odor headspace (Table 1). Individual mice differed in their sensitivity to this odorant by less than 1 order of magnitude ( $0.8\text{--}2.8 \times 10^{-10}$  M) and there was no sex difference observed in 1-propanol sensitivity ( $P = 0.55$ ,  $F = 0.35$ , sum of squares test).

Mice were less sensitive to 1-butanol (~35 fold), responding with a threshold of  $4.5 \times 10^{-9}$  M (95% CI:  $1.2\text{--}6.3 \times 10^{-9}$ ) or 0.108 ppm ( $P < 0.001$ ,  $F = 72.96$ , sum of squares test; Figure 2b,f; Table 1). This concentration is equivalent to a  $2.1 \times 10^{-6}$  (95% CI:  $1.0\text{--}4.3 \times 10^{-6}$ ) liquid dilution of 1-butanol in water (v/v) with a 10% air dilution of the odor headspace (Table 1). Individual mice exhibited slightly more variable responses to this odorant differing by more than 1 order of magnitude ( $0.8\text{--}242 \times 10^{-9}$  M). Similar to 1-propanol, we did not observe any sex differences in 1-butanol sensitivity ( $P = 0.14$ ,  $F = 2.18$ , sum of squares test).

Mice were more sensitive to 1-pentanol (~20 fold) than 1-butanol as their threshold to 1-pentanol was  $2.3 \times 10^{-10}$  M (95% CI:  $1.2\text{--}4.2 \times 10^{-10}$ ) or 0.006 ppm ( $P < 0.001$ ,  $F = 38.37$ , sum of squares test; Figure 2c,f; Table 1). However, the animal's sensitivity to 1-propanol and 1-pentanol did not differ ( $P = 0.1124$ ,  $F = 2.6$ , sum of squares test; Figure 2f). The 1-pentanol threshold was equivalent to  $8.0 \times 10^{-8}$  (95% CI:  $4.3\text{--}14.9 \times 10^{-8}$ ) liquid dilution of 1-pentanol in water (v/v) with a 10% air dilution of the odor headspace. With the exception of 1 outlier ( $4.1 \times 10^{-9}$  M), individual mice exhibited very consistent thresholds ( $1.0\text{--}3.3 \times 10^{-10}$  M) and there was no sex difference in 1-pentanol sensitivity ( $P = 0.77$ ,  $F = 0.08$ , sum of squares test).

Mice were most sensitive to 1-hexanol as they had a behavioral threshold of  $3.0 \times 10^{-11}$  M (95% CI:  $1.7\text{--}5.3 \times 10^{-11}$ ) or 0.0006 ppm (Figure 2d,f; Table 1). Specifically, mice were more sensitive to 1-hexanol than 1-propanol (~4 fold), 1-butanol (~150 fold), 1-pentanol (~7 fold), and even 1-heptanol (~4 fold;  $P < 0.0001$ ,  $F = 24.21$ , sum of squares test; Figure 2f; Table 1). The 1-hexanol threshold is equivalent to a  $1.2 \times 10^{-8}$  (95% CI:  $0.7\text{--}1.9 \times 10^{-8}$ ) dilution of 1-hexanol in water (v/v) with a 10% air dilution of the odor headspace. Individual mice differed in their sensitivity to 1-hexanol by more than 1 order of magnitude ( $0.8\text{--}116 \times 10^{-11}$  M<sup>0</sup>) but did not differ by sex ( $P = 0.11$ ,  $F = 2.64$ , sum of squares test).

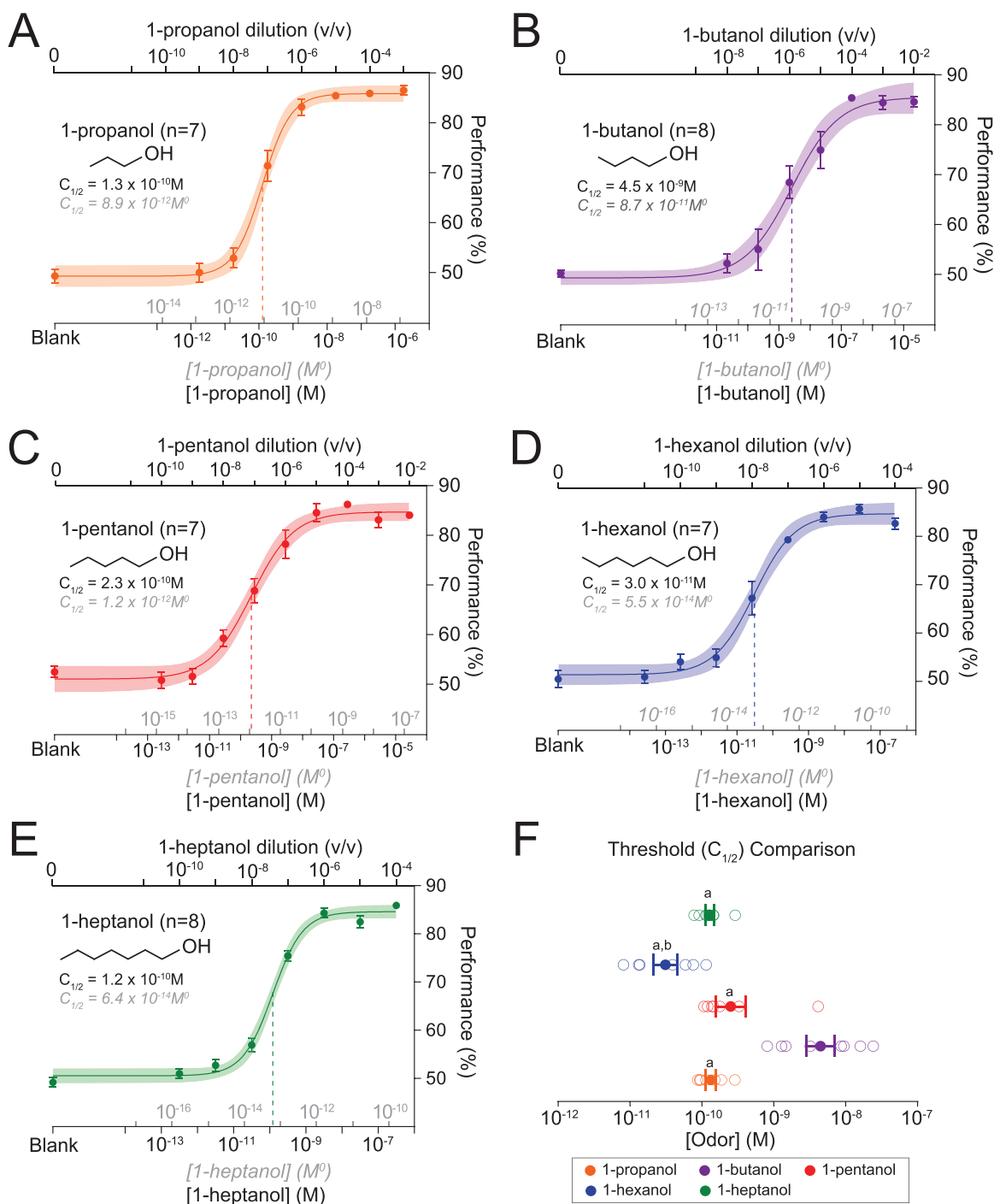
The vapor phase detection threshold for 1-heptanol was  $1.2 \times 10^{-10}$  M (95% CI:  $0.9\text{--}1.7 \times 10^{-10}$ ) or 0.002 ppm (Figure 2e). This threshold was lower than 1-butanol (~35-fold;  $P < 0.0001$ ,  $F = 99.6$ , sum of squares test) but statistically indistinguishable from either 1-propanol or 1-pentanol ( $P > 0.05$ ,  $F = 3.2$ , sum of squares test; Figure 2e,f; Table 1). These results are equivalent to a  $4.0 \times 10^{-8}$  (95% CI:  $2.9\text{--}5.3 \times 10^{-8}$ ) dilution of 1-heptanol in water (v/v) with a 10% air dilution of the odor headspace. Individual mice were extremely similar in their sensitivity to 1-heptanol differing by less than 1 order of magnitude ( $0.8\text{--}2.9 \times 10^{-10}$  M). Similar to all the other odorants tested, we did not observe any sex differences in 1-heptanol sensitivity ( $P = 0.66$ ,  $F = 0.19$ , sum of squares test).

Accounting for the odorant's activity in water, we found no statistically significant correlations between gas vapor phase detection thresholds and either carbon chain length ( $r_s = -0.544$ ;  $P = 0.172$ , Spearman correlation; Figure 3) or the volatility of the odorant ( $r_s = 0.245$ ;  $P = 0.345$ , Spearman correlation; Table 2). However, we found a statistically significant negative correlation between the ideal gas vapor phase detection thresholds and carbon chain length ( $r_s = -0.859$ ;  $P = 0.031$ , Spearman correlation) but not the volatility of the odorant ( $r_s = 0.615$ ;  $P = 0.135$ , Spearman correlation; Table 2). Liquid dilution thresholds were not correlated with either the length of the alcohol carbon chain ( $r_s = -0.544$ ;  $P = 0.172$ , Spearman correlation) or its volatility ( $r_s = 0.245$ ,  $P = 0.345$ ; Spearman correlation; Table 2).

## Discussion

We found that C57Bl/6J mice are very sensitive to primary aliphatic alcohols, reliably detecting concentrations below  $10^{-8}$  M (or  $10^{-10}$  M<sup>0</sup>). Interestingly, there are large differences in the behavioral sensitivity observed across these structurally similar alcohols as mice are approximately 150-fold more sensitive to 1-hexanol than 1-butanol. Sensitivity to these different alcohols did not differ by sex and were relatively consistent across individuals. Similar to previous studies, we also observed that differences in sensitivity are correlated with carbon chain length in an ideal gas condition. However, at least in mice, this correlation appears to be heavily influenced by the odorant/solvent interactions. In summary, our approach puts forth robust estimates of behavioral sensitivity that will hopefully guide experimenters in choosing appropriate concentrations for functional studies in mice using these odorants.

Estimates of behavioral sensitivity can be influenced by a number of factors including the behavioral assay, method of odor delivery, strain tested, definition of threshold, and the solvent used (Bodyak and Slotnick 1999; Slotnick and Schellinck 2002; Tsukani et al. 2003; Slotnick and Restrepo 2005; Laska 2015). Methods such as the 2-bottle preference test (e.g., D'Hulst et al. 2016), maze learning (e.g., Sato et al. 2015), and odor investigation assays (e.g., Sato-Akumura et al. 2016) have been used to compare odor sensitivity between experimental animals but are not optimal for determining odor detection thresholds (Bodyak and Slotnick 1999; Slotnick and Schellinck 2002). Instead, psychophysical analyses using operant conditioning procedures and flow-dilution olfactometers are ideally suited to define odor detection thresholds. However, these experiments can still differ in their estimate of threshold by many orders of magnitude. Our method has resulted in estimates of behavioral sensitivity that are both internally consistent (across individuals and different cohorts) and are some of the lowest reported for any rodent species thus far. Accordingly, our approach also differs from other studies in a variety of aspects (see Methods), including several that



**Figure 2.** C57BL/6J mice are very sensitive to primary aliphatic alcohols. Psychometric curves to 1-propanol (A), 1-butanol (B), 1-pentanol (C), 1-hexanol (D), 1-heptanol (E), and a summary of all odorants (F). Data were fitted using a hill function. Maximal behavioral performance for each odor concentration is limited to ~85% (see Methods). Behavioral threshold ( $C_{1/2}$ ) is demarcated with a dashed line (A–E). Plots show mean  $\pm$  SE with shaded 95% CI. x axis includes the odor concentration according to the ideal gas condition (gray) and the nonideal gas condition (black). Top x axis denotes the liquid dilution tested in the olfactometer. (F) Summary of behavioral sensitivity for all odorants. Plots show mean  $\pm$  SE. Individual thresholds for each odorant are denoted with an open circle. a—signifies a statistical difference from the 1-butanol threshold ( $P < 0.05$ , sum of squares test). b—signifies a statistical difference from the 1-propanol, 1-pentanol, and 1-heptanol thresholds ( $P < 0.05$ , sum of squares test).

require further discussion. First, the strain of mice tested (C57Bl/6J) differed from other studies. It is likely that strain differences contribute to some of the variations in behavioral threshold estimates across studies. In fact, C57Bl/6J mice are known to differ in their olfactory discrimination learning ability as compared with an outbred strain (CD-1) of mice (Laska 2015). These potential differences

underlie the importance of threshold measures in a commonly used inbred strain of mice, such as C57Bl/6J, to ensure applicability to a wide range of studies. Second, threshold criterion can vastly differ between studies. Another common approach is to use binomial statistics to determine the concentration that is significantly discriminated above chance level (e.g., Laska et al. 2006; Lotvedt et al.

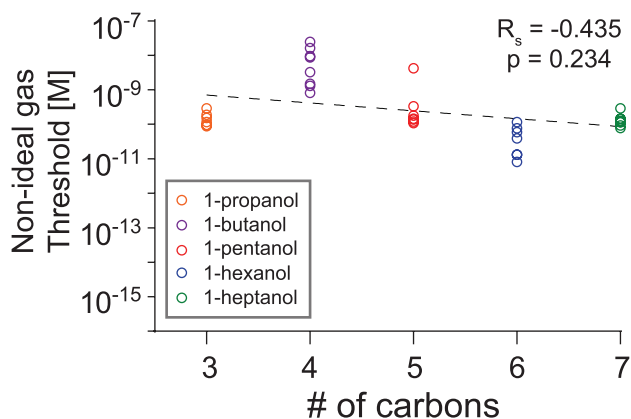
**Table 1.** Olfactory detection thresholds of C57Bl/6J mice to primary aliphatic alcohols

| Odorant    | $\gamma$          | Dilution             | Ideal gas condition |                       | Nonideal gas condition |                       |
|------------|-------------------|----------------------|---------------------|-----------------------|------------------------|-----------------------|
|            |                   |                      | ppm <sup>0</sup>    | M <sup>0</sup>        | ppm                    | M                     |
| 1-propanol | 14.2 <sup>a</sup> | $7.1 \times 10^{-8}$ | 0.0002              | $8.9 \times 10^{-12}$ | 0.003                  | $1.3 \times 10^{-10}$ |
| 1-butanol  | 51.3 <sup>a</sup> | $2.1 \times 10^{-6}$ | 0.0021              | $8.7 \times 10^{-11}$ | 0.108                  | $4.5 \times 10^{-9}$  |
| 1-pentanol | 195 <sup>a</sup>  | $8.0 \times 10^{-8}$ | 0.00003             | $1.2 \times 10^{-12}$ | 0.006                  | $2.3 \times 10^{-10}$ |
| 1-hexanol  | 552 <sup>b</sup>  | $1.2 \times 10^{-8}$ | 0.000001            | $5.5 \times 10^{-14}$ | 0.0006                 | $3.0 \times 10^{-11}$ |
| 1-heptanol | 1970 <sup>b</sup> | $4.0 \times 10^{-8}$ | 0.000001            | $6.4 \times 10^{-14}$ | 0.002                  | $1.2 \times 10^{-10}$ |

References for activity coefficient ( $\gamma$ ) in water are as follows:

<sup>a</sup>Dohnal et al. 2006.

<sup>b</sup>Tochigi et al. 2000.



**Figure 3.** Alcohol sensitivity and carbon chain length are not statistically correlated. The olfactory threshold of individual mice to aliphatic alcohols (C3–C7) is plotted. The relationship between olfactory threshold and carbon chain length is compared with a Spearman's correlation.

2012). Using this approach, our data yielded behavioral thresholds that were roughly 2- to 100-fold lower (i.e., more sensitive) than our  $C_{1/2}$  estimates of behavioral sensitivity—highlighting this potential source of variability across studies. Third, we only tested mice on 1 odor concentration per day. We favor this more time-consuming approach because it prevents contamination of the common pathways of the olfactometer by higher concentrations of the target odor. This is seemingly advantageous because the mouse's ability to detect low (near threshold) concentrations cannot be directly masked by residual contamination or indirectly masked by adaptation resulting from the presentation of higher concentrations of the target odor. Lastly, we have included a cheating check (see Methods) into our approach that tests for the ability of mice to use nonodor cues to maximize performance. This potential confound has been noted in previous studies (Clevenger and Restrepo 2006; Laska et al. 2008; Dewan et al. 2018); however, our approach provides a method to assess the degree to which it is occurring. Thus, each session included in our data set presumably lacks these artificial boosts in performance and represents our best estimates of detection threshold for this strain of mice.

In general, our updated threshold measures have considerably lowered the estimates of murine sensitivity for these odorants. However, it should be noted that previous studies used a wide array of different solvents and, in most cases, do not account for the odorant/solvent interactions. In fact, our estimates of sensitivity differed by several orders of magnitude, once the odorant/solvent interactions were factored into our analyses. Unfortunately, similar

**Table 2.** Correlation between olfactory detection thresholds and either carbon chain length or odorant volatility

|                     | Ideal gas threshold [M <sup>0</sup> ] | Nonideal gas threshold [M] | Liquid dilution threshold (v/v) |
|---------------------|---------------------------------------|----------------------------|---------------------------------|
| Carbon chain length | <b>-0.8599*</b>                       | -0.5440                    | -0.4345                         |
| Volatility (mmHg)   | 0.6419                                | 0.2454                     | 0.1317                          |

Spearman's rank correlation coefficient ( $R_s$ ) are listed. Significant correlations are bolded and denoted by \* ( $P < 0.05$ ).

corrections were not possible for all previous estimations of alcohol sensitivity as the appropriate activity coefficients are not available for all solvents. Thus, we have attempted to compare our measures of sensitivity to the rodent literature using the more accurate nonideal gas thresholds whenever possible and noting the solvent used in each study. Threshold estimates of 1-propanol in mice range from  $\sim 1.8 \times 10^{-7}$  M (water) to  $\sim 3.0 \times 10^{-8}$  M (only flow dilution; Youngentob and Margolis 1999; Pho et al. 2005). These thresholds are more than 100-fold higher than the nonideal gas threshold ( $1.3 \times 10^{-10}$  M) of C57Bl/6J mice. In contrast, Smith et al. (2008) reported 1-propanol thresholds of  $2.3 \times 10^{-13}$  M<sup>0</sup> (mineral oil), which is lower than our ideal gas measures of threshold ( $8.9 \times 10^{-12}$  M<sup>0</sup>) for this odorant. Prior estimates for 1-butanol sensitivity ( $5.5 \times 10^{-4}$  to  $4.0 \times 10^{-7}$  M in water; Deiss and Baudoin 1997; Larson et al. 2003) are also suitably higher than our nonideal gas threshold of  $4.5 \times 10^{-9}$  M for this odorant. Lotvedt et al. (2012) determined that the threshold of CD-1 mice to 1-hexanol was  $1.3\text{--}13 \times 10^{-10}$  M<sup>0</sup> (diethyl phthalate), more than 1000-fold higher than our ideal gas estimate of sensitivity ( $5.5 \times 10^{-14}$  M<sup>0</sup>) in C57Bl/6J mice. Lastly, our estimate of 1-heptanol sensitivity ( $6.4 \times 10^{-14}$  M<sup>0</sup>) is also significantly lower than prior estimates in mice (mineral oil,  $4 \times 10^{-8}$  M<sup>0</sup>; Smith et al. 2008). In rats, a systematic study of alcohol sensitivity reported thresholds that ranged from  $1.0 \times 10^{-6}$  M<sup>0</sup> for 1-propanol to  $1.5 \times 10^{-9}$  M<sup>0</sup> for 1-heptanol (propylene glycol and water; Moulton and Eayrs 1960), approximately 5–6 orders of magnitude less sensitive than our ideal gas thresholds in C57Bl/6J mice. However, later studies significantly lowered these estimates for specific aliphatic alcohol in the rat (Laing 1975; Youngentob et al. 1997; Yoder et al. 2017). In summary, although it is possible that threshold disparities are solely due to the receptor repertoire of these different strains or species, it is probably more likely that different approaches allow the experimenter to more or less accurately capture the lower limits of detection.

Previous studies have suggested that behavioral sensitivity is negatively correlated with the carbon chain length of the alcohol. Although this relationship was not previously tested in mice, it appears to be present in rats (solvent: propylene glycol and water;

Moulton and Eayrs 1960), several nonhuman primates (solvent: diethyl phthalate; Laska et al. 2006; Laska and Seibt 2002) and even humans (solvent: mineral oil; Cometto-Muniz and Cain 1990). In fact, similar negative correlations have also been found for homologous series of other odorants, including aliphatic carboxylic acids (solvent: diethyl phthalate; Laska et al. 2000; Can Guven and Laska 2012) and acetic esters (solvent: diethyl phthalate; Laska and Seibt 2002). We found that, at least in mice, the correlation between carbon chain length and alcohol sensitivity can be explained by the activity of the odorant in the solvent and is not due to differences in sensitivity. It is unclear whether the observed relationships between carbon chain length and odorant sensitivity in these other species are also influenced by solvent effects to the same degree.

Our finding that mice are differentially sensitive to each aliphatic alcohol has implications for functional experiments. Panels of structurally similar odorants are typically presented at equivalent liquid dilutions or even vapor-matched concentrations to map odor-evoked responses throughout the olfactory system (e.g., Uchida et al. 2000; Takahasi et al. 2004). Although the exact relationship between odor-evoked responses and perception is unclear, our results indicate that mice differ in their ability to detect vapor-matched or equivalent liquid dilutions of these alcohols. Thus, it is possible that the perceived intensity of these stimuli may differ even among concentration-matched, structurally similar odorants. This observation could complicate the interpretation of odor mapping studies, as well as odor-guided behavioral tests that use aliphatic alcohols, such as measures of innate odor aversion or odor discrimination assays.

In addition to the main olfactory system, several receptor systems in the nasal cavity can detect airborne chemicals and, therefore, have the potential to impact detection threshold. Of note, the trigeminal system of the rat is responsive to high concentrations of aliphatic alcohols (Silver et al. 1986). These neural response thresholds (determined by delivering odor via an air-dilution olfactometer and recording from the ethmoid nerve) were measured at between 40 ppm for 1-heptanol and 1500 ppm for 1-propanol (Silver et al. 1986). Although these data are unavailable in the mouse, it should be noted that these stimulus concentrations exceed most of the concentrations tested in our study. Thus, it is unlikely that mice are using their trigeminal system to enhance their sensitivity to alcohols.

In summary, we have provided robust estimates of aliphatic alcohol sensitivity in C57Bl/6J mice (the most commonly used inbred strain). These estimates have significantly lower the presumed detection threshold of the species and likely indicate that some functional studies may be employing higher than ideal odor concentrations. We also observed dramatic differences in the sensitivity to various aliphatic alcohols. This observation has functional implications as additional perceptual differences may exist for equivalent liquid dilutions or even vapor-matched concentrations of structurally similar odorants. This is particularly important because the potential correlation between alcohol sensitivity and carbon chain length (at least in mice) appears to be due to solvent effects and not detection ability. Thus, effective concentrations for functional studies in mice cannot be predicted based on carbon chain length and should instead rely on sensitivity measures, such as those employed in the current study.

## Supplementary material

Supplementary material can be found at *Chemical Senses* online.

Figure S1. Example lick responses from an individual mouse for the first 300 trials tested with 1-butanol ( $2.17 \times 10^{-6}$  M). Initial go trials are not included. The behavioral responses according to trial type are marked with different colors and symbols. The behavioral response (correct go or false alarm)

of the animal was determined with the first lick (see methods); however, the subsequent licks for that trial are similarly colored. Licks associated with a correct go trial (151/152) are denoted with black squares. The water reward immediately followed this behavioral response and the subsequent licks are the mouse receiving this reward. A single red X denotes the only missed go trial. Correct nogo responses (110/148) lack licks and can be visualized by the absence of any symbols. False alarms associated with cheating check nogo trials are denoted with green squares (35 trials) while false alarms during the presentation of the odor (nogo trials) are denoted with red squares (3 trials). The dashed line indicates the approximate time to max stimulus concentration (see Figure 1b).

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## Conflict of interests

The authors have no conflict of interests.

## References

- Berdichevskaia A, Caze R, Schultz S. 2016. Performance in a GO/NOGO perceptual task reflects a balance between impulsive and instrumental components of behaviour. *Sci Rep.* 6:27389.
- Bodyak N, Slotnick B. 1999. Performance of mice in an automated olfactometer: odor detection, discrimination and odor memory. *Chem Senses.* 24:637–645.
- Can Guven S, Laska, M. 2012. Olfactory sensitivity and odor structure-activity relationships for aliphatic carboxylic acids in CD-1 mice. *PLoS One.* 7:E34301
- Clevenger A, Restrepo D. 2006. Evaluation of the validity of maximum likelihood adaptive staircase procedure for measurement of olfactory detection threshold in mice. *Chem Senses.* 31:9–26.
- Cometto-Muñiz JE, Cain WS. 1990. Thresholds for odor and nasal pungency. *Physiol Behav.* 48(5):719–725.
- Deiss V, Baudoin C. 1997. Hyposmia for butanol and vanillin in mutant staggerer male mice. *Physiol Behav.* 61(2):209–213.
- Dewan A, Cichy A, Zhang J, Miguel K, Feinstein P, Rinberg D, Bozza T. 2018. Single olfactory receptors set odor detection thresholds. *Nat Commun.* 9(1):2887.
- D'Hulst C, Mina RB, Gershon Z, Jamet S, Cerullo A, Tomoiaga D, Bai L, Belluscio L, Rogers ME, Sirotnin Y, et al. 2016. MouSensor: a versatile genetic platform to create super sniffer mice for studying human odor coding. *Cell Rep.* 16(4):1115–1125.
- Dohnal V, Fenclova D, Vrbka P. 2006. Temperature dependences of limiting activity coefficients, Henry's Law constants, and derivative infinite dilution properties of lower (C1-C5) 1-alkanols in water. Critical compilation, correlation, and recommended data. *J Phys Chem.* 35:1621–1651.
- Harvey L. 1986. Efficient estimation of sensory thresholds. *Behav Res Methods Instrum Comput.* 18:623–632.
- Inaki K, Takahashi YK, Nagayama S, Mori K. 2002. Molecular-feature domains with posterodorsal-anteroventral polarity in the symmetrical sensory maps of the mouse olfactory bulb: mapping of odourant-induced Zif268 expression. *Eur J Neurosci.* 15(10):1563–1574.
- Iurilli G, Datta SR. 2017. Population coding in an innately relevant olfactory area. *Neuron.* 93(5):1180–1197.e7.



- Johnson BA, Xu Z, Ali SS, Leon M. 2009. Spatial representations of odorants in olfactory bulbs of rats and mice: similarities and differences in chemotopic organization. *J Comp Neurol*. 514(6):658–673.
- Kauer JS, White J. 2001. Imaging and coding in the olfactory system. *Annu Rev Neurosci*. 24:963–979.
- Laing D. 1975. A comparative study of the olfactory sensitivity of humans and rats. *Chem Senses*. 1:257–269.
- Larson J, Hoffman JS, Guidotti A, Costa E. 2003. Olfactory discrimination learning deficit in heterozygous reeler mice. *Brain Res*. 971(1):40–46.
- Laska M. 2015. Olfactory discrimination learning in an outbred and an inbred strain of mice. *Chem Senses*. 40(7):489–496.
- Laska M, Joshi D, Shepherd GM. 2006. Olfactory sensitivity for aliphatic aldehydes in CD-1 mice. *Behav Brain Res*. 167(2):349–354.
- Laska M, Rivas Bautista RM, Hernandez Salazar LT. 2006. Olfactory sensitivity for aliphatic alcohols and aldehydes in spider monkeys (*Ateles geoffroyi*). *Am J Phys Anthropol*. 129(1):112–120.
- Laska M, Rosandher A, Hommen S. 2008. Olfactory discrimination of aliphatic odorants at 1ppm: too easy for CD-1 mice to show odor structure-activity relationships? *J Comp Physiol*. 194:971–980.
- Laska M, Seibt A. 2002. Olfactory sensitivity for aliphatic alcohols in squirrel monkeys and pigtail macaques. *J Exp Biol*. 205(Pt 11):1633–1643.
- Laska M, Seibt A, Weber A. 2000. “Microsmatic” primates revisited: olfactory sensitivity in the squirrel monkey. *Chem Senses*. 25(1):47–53.
- Liu G, Patel J, Tepe B, McClard C, Swanson J, Quast K, Arenkiel B. 2018. An objective and reproducible test of olfactory learning and discrimination in mice. *J Vis Exp*. 133:57142.
- Lotvedt P, Murali S, Salazar L, Laska M. 2012. Olfactory sensitivity for “green odors” (aliphatic C6 and C6 aldehydes)—a comparative study in male CD-1 mice (*Mus musculus*) and female spiked monkeys (*Ateles geoffroyi*). *Pharmacol Biochem Behav*. 101:450–457.
- Malnic B, Hirono J, Sato T, Buck LB. 1999. Combinatorial receptor codes for odors. *Cell*. 96(5):713–723.
- Moulton DG, Eayrs JT. 1960. Studies in olfactory acuity. II: relative detectability of n-aliphatic alcohols by the rat. *Q J Exp Psychol*. 12(2):99–109.
- Pho V, Butman ML, Cherry JA. 2005. Type 4 phosphodiesterase inhibition impairs detection of low odor concentrations in mice. *Behav Brain Res*. 161(2):245–253.
- Sato T, Hirono J, Tonoike M, Takebayashi M. 1994. Tuning specificities to aliphatic odorants in mouse olfactory receptor neurons and their local distribution. *J Neurophysiol*. 72(6):2980–2989.
- Sato T, Kobayakawa R, Kobayakawa K, Emura M, Itohara S, Kizumi M, Hamana H, Tsuboi A, Hirono J. 2015. Supersensitive detection and discrimination of enantiomers by dorsal olfactory receptors: evidence for hierarchical odour coding. *Sci Rep*. 5:14073.
- Sato-Akuhara N, Horio N, Kato-Namba A, Yoshikawa K, Niimura Y, Ihara S, Shirasu M, Touhara K. 2016. Ligand specificity and evolution of mammalian musk odor receptors: effect of single receptor deletion on odor detection. *J Neurosci*. 36(16):4482–4491.
- Shusterman R, Smear MC, Koulakov AA, Rinberg D. 2011. Precise olfactory responses tile the sniff cycle. *Nat Neurosci*. 14(8):1039–1044.
- Silver WL, Mason JR, Adams MA, Smeraski CA. 1986. Nasal trigeminal chemoreception: responses to n-aliphatic alcohols. *Brain Res*. 376(2):221–229.
- Slotnick B, Restrepo D. 2005. Olfactometry with mice. *Curr Protoc Neurosci*. Chapter 8:Unit 8.20.
- Slotnick B, Schellinck H. 2002. Methods in olfactory research with rodents. In: Simon SA, Nicolelis M, editors. *Frontiers and methods in chemosenses*. Boca Raton (FL): CRC. p. 21–61.
- Smear M, Resulaj A, Zhang J, Bozza T, Rinberg D. 2013. Multiple perceptible signals from a single olfactory glomerulus. *Nat Neurosci*. 16(11):1687–1691.
- Smith DW, Thach S, Marshall EL, Mendoza MG, Kleene SJ. 2008. Mice lacking NKCC1 have normal olfactory sensitivity. *Physiol Behav*. 93(1–2):44–49.
- Takahashi YK, Kurosaki M, Hirono S, Mori K. 2004. Topographic representation of odorant molecular features in the rat olfactory bulb. *J Neurophysiol*. 92(4):2413–2427.
- Tochigi K., Uchiyama M, Kojima K. 2000. Measurement of infinite-dilution activity coefficients of alcohols in water using relative gas-liquid chromatographic method. *Korean J Chem Eng*. 17:502–505.
- Tsukatani T, Miwa T, Furukawa M, Costanzo RM. 2003. Detection thresholds for phenyl ethyl alcohol using serial dilutions in different solvents. *Chem Senses*. 28(1):25–32.
- Uchida N, Takahashi YK, Tanifuji M, Mori K. 2000. Odor maps in the mammalian olfactory bulb: domain organization and odorant structural features. *Nat Neurosci*. 3(10):1035–1043.
- Xu W, Wilson DA. 2012. Odor-evoked activity in the mouse lateral entorhinal cortex. *Neuroscience*. 223:12–20.
- Yoder WM, Gaynor LS, Burke SN, Setlow B, Smith DW, Bizon JL. 2017. Interaction between age and perceptual similarity in olfactory discrimination learning in F344 rats: relationships with spatial learning. *Neurobiol Aging*. 53:122–137.
- Youngentob SL, Margolis FL. 1999. OMP gene deletion causes an elevation in behavioral threshold sensitivity. *Neuroreport*. 10(1):15–19.
- Youngentob SL, Schwob JE, Sheehe PR, Youngentob LM. 1997. Odorant threshold following methyl bromide-induced lesions of the olfactory epithelium. *Physiol Behav*. 62(6):1241–1252.