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Original Article

Transfer of Odor Perception From the Retronasal to the Orthonasal Pathway

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Abstract

Although orthonasal odorants are often associated with the external environment, retronasal odorants are accompanied by consummatory behaviors and indicate an internal state of an animal. Our study aimed to examine whether the same odorants may generate a consistent perceptual experience when 2 olfactory routes potentiate variations in concentration in the nasal cavity and orosensory activation. A customized lick spout with vacuum removing odorants around the animal's nares was used to render a pure retronasal exposure experience. We found that pre-exposing rats to odorants retronasally with positive or negative reinforcers (sweet or bitter) lead to a significant learning rate difference between high- and low-vapor-pressure odorants. This effect was not observed for novel odorants, suggesting that odorants may generate similar perceptual quality in a volatility-dependent manner.

Key words: retronasal olfaction, learning, odor discrimination

Smelling occurs via 2 routes in mammals. Odorant molecules enter the nasal cavity orthonasally as an indication of objects presented in the external environment. The molecules can also volatilize retronasally from the nasopharynx, carrying information about food intake and internal status (Figure 1a). Rozin (1982) observed the discrepancy between the 2 olfactory routes in terms of pleasantness and intensity ratings of items such as cheese and fish and proposed the duality of olfaction, that the same odorant can generate qualitatively different percepts depending on the route it takes. Consistent with Rozin's hypothesis, Hannum et al. (2018) show that participants are worse at matching an unfamiliar or a similar odor reference to test odorants when the reference and test odorants are presented via the 2 different routes, suggesting that the 2 routes evoke different percepts.

Retronasal and orthonasal olfaction involve distinctive behavioral patterns (sniffing, chewing, licking, etc.) that affect the time course and air flow of an olfactory input and recruit other sensory modalities including taste and somatosensation. In humans, this leads to quantitative differences in detection threshold, hedonic rating, adaptation, and difficulty in localization of odorants depending on the route (Pierce and Halpern 1996; Hummel and Livermore 2002; Hummel et al. 2006). However, the chemical profile for an odorant remains the same regardless of the route and activates a similar set of olfactory sensory neurons and glomerular activation patterns in the olfactory bulbs (Gautam and Verhagen 2012; Rebello et al. 2015). It is intriguing to understand how the same sensory input may lead to overlapping or diverging percepts and neural responses in the olfactory system when entering from 2 routes. An EEG study in humans showed that context and familiarity of an odorant interact with the source of the odorant (orthonasal or retronasal) to affect both the perceptual intensity and the odor-event-related potential (Hummel and Heilmann 2008). Small et al. (2005) used fMRI and demonstrated a stronger blood-oxygen-level-dependent response in the amygdala, hippocampus, and insula when odors are delivered orthonasally. Retronasally delivered odors, on the other hand, activated the central sulcus, a brain region often associated with orosensory input.

Nonhuman animal models allow researchers to examine the early anatomical process of olfactory inputs and cross modality interactions. Although both retronasal and orthonasal olfaction activate a similar ensemble of glomeruli, retronasal olfaction evokes a weaker response in the olfactory bulb and has a longer

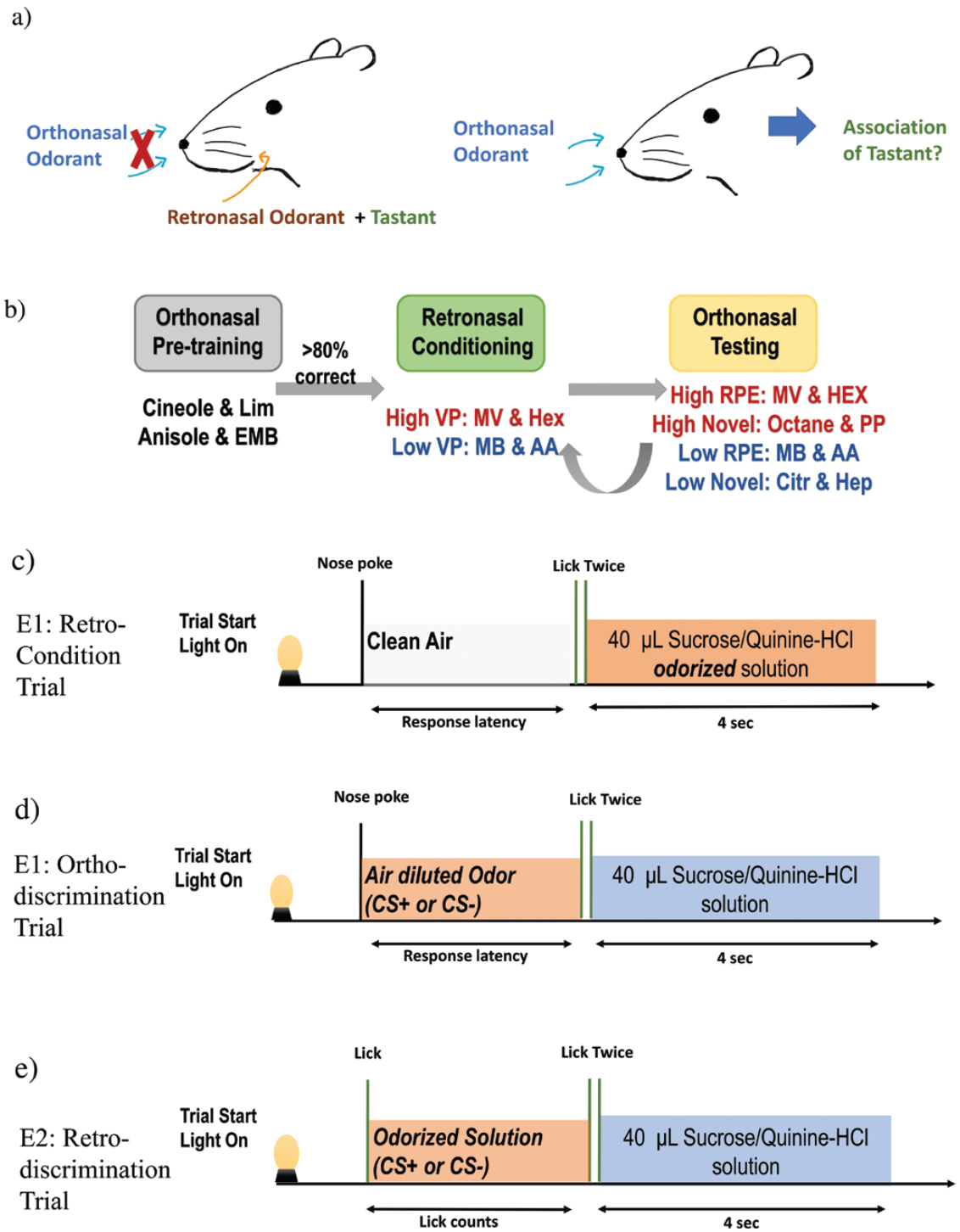

Figure 1. Schematics of delivery routes and experiment procedures. (**a**) The study tests whether rats can recall a learned association between a purely retronasal odorant and tastant when subsequently exposed to the odorant orthonasally. (**b**) Experiment 1 consists of the retronasal conditioning phase and orthonasal testing phase. After being pretrained to perform the orthonasal discrimination task with 2 odor sets, rats were transferred to 2 days of retronasal conditioning and 2 days of orthonasal testing (150 trials per session, 1 session per day) (lim, limonene; EMB, ethyl 2-methylbutyrate; MV, methyl valerate; Hex, hexanone; PP, propyl propionate; MB, methyl benzoate; AA, amyl acetate; Citron, citronelle; Hep, heptanol). (**c**) During a retronasal conditioning trial, a rat receives clean air when nose poking in the odor port and licks the spout twice to receive odor+taste solution. Orthonasal odorants are removed via a concentric vacuum. (**d**) A rat is tested on the orthonasal discrimination task where odorized air is delivered, and the rat chooses to either lick or not lick for the reinforcer solution associated with the odorant. (**e**) Experiment 2 examines rats' ability to discriminate retronasal odorants. Dispensing of odorized solution is initiated by one lick, and if the rat continues licking (more than 2 licks), the sweet or bitter reinforcer is delivered.

response delay (Gautam and Verhagen 2012; Furudono et al. 2013). Collectively, the evidence is mixed on whether the retronasal and orthonasal routes share common percepts. Different interpretations are based on speculation regarding how odorants are encoded in the neural system. On the one hand, at the peripheral level, the olfactory sensory receptors' responses and the glomerular activation

patterns within the olfactory bulb overlap highly between routes, which supports the claim of shared percepts between the routes. On the other hand, multisensory, temporal, and intensity differences at the periphery and in functional network recruitment may potentiate the perceptual difference between the routes.

The importance of olfactory route studies lies in understanding the role that the different routes play in the learning process, where the information gained from one route should be effectively shared with the other in flavor acquisition. Chapuis et al. (2007) showed that although odor presented near water alone (i.e., orthonasally) is sufficient to induce conditioned food aversion with LiCl injection in rats, adding the odorant into the water (both retronasally and orthonasally) elicits a stronger aversive response that is also more resistant to extinction. This additive effect is consistent with a technique used to accelerate odor–taste association acquisition (Darling and Slotnick 1994; Kay and Laurent 1999). Blankenship et al. (2019) showed that rats learn faster and in fewer days to preferentially respond to positively reinforced retronasal odorants compared with orthonasal odorants. With the learning paradigm used in the experiment, the animals showed no transfer of preference between orally infused odorants to airborne odorants. Together, these results suggest that although olfactory learning may arise from both routes, learning strength and efficiency and perceptual quality may differ significantly across olfactory routes.

To investigate whether retronasal and orthonasal routes generate qualitatively similar percepts, we pre-exposed rats to both appetitive and aversive odor–taste associations in a pure retronasal experience. We hypothesized that if the 2 olfactory routes generate similar percepts, retronasal learning should transfer to orthonasal recognition. We predicted that rats would perform better or learn faster to orthonasally discriminate retronasally pre-exposed (RPE) odor sets compared with novel odor sets. Because olfactory learning can be affected by the strength of the stimulus, we use both high- and low-volatility odorants. We show that learning transfer from pure retronasal experience to orthonasal odor discrimination is possible for the high-volatility odorants, suggesting that the 2 olfactory routes can share similar percepts if the odors are strong enough.

Materials and methods

Subjects

Twenty Sprague-Dawley male rats purchased from Envigo were used for 2 experiments (6 weeks old upon arrival with 6–8 weeks after arrival until experiments were concluded; 16 rats for Experiment 1, 4 rats for Experiment 2). Rats were pair housed in transparent home cages in a vivarium maintained on a 14-h light cycle (lights on 08:00–22:00 CST) with unlimited access to food and water until they began the experiment. Experiments were performed during the light phase to avoid providing light to the rats during their dark phase because dark phase light can alter circadian rhythms and have significant negative outcomes on health and cognition (Travlos et al. 2001; Bedrosian et al. 2013). Two weeks prior to the training phase, the rats were separated and singly housed, and each animal was moved over several days to a 23-h water deprivation protocol with ad libitum water for one hour at the same time every day. Each rat was tested and given water at the same time each day, which favors entrainment to the test time (Dhume and Gogate 1982; Mistlberger 1992). All subjects were weighed and handled daily until water consumption was stable (~30–40 mL/day). All procedures were performed under veterinary supervision and approved by the University of Chicago Institutional Animal Care and Use Committee in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care.

Apparatus

Experiments were conducted in an operant conditioning modular test chamber (ENV-008, Med Associates, Georgia, VT) and programmed with MedPC-IV software. The end walls were made of aluminum. In the front of the chamber, an odor port equipped with an infrared beam sensor recorded the start time and duration of each entry. A house light located at the back of the box was illuminated at the beginning of each trial to signify the opening of the door at the front of the box, which granted access to the odor port. The door was adapted from a CD ROM drive and programmed by an Arduino driver. A lick spout with a concentric vacuum was located inside the odor port and connected to a vacuum line adjacent to the liquid dispensers. The vacuum was turned on during the retronasal conditioning phase to remove orthonasal odorants, rendering the experience purely retronasal (Rebello et al. 2015). Solenoid isolation valves (NResearch 225T012) mounted on the wall with separate tubing (diameter 1.5 mm) dispensed ~40 µL of liquid each time they were activated in a trial. Licking was measured by an infrared photobeam lickometer located in front of a lick spout. The odor (i.e., retronasal) and taste solutions were delivered through separate odor delivery tubing and separate internal tubing in the lick spout to avoid cross-contamination. Odorants in the orthonasal condition were diluted at approximately 15% with air and delivered using our standard protocol (Kay and Beshel 2010; Frederick et al. 2017).

Behavioral protocols

E1: Training schedule

Water-restricted rats were conditioned to an odorized sweet solution and an odorized bitter solution in the retronasal phase and tested on the orthonasal odor discrimination tasks to discriminate either the pre-exposed odor pair or a novel odor pair matched for volatility (Figure 1b). Rats learn the instrumental orthonasal discrimination task with some degree of variability across subjects, and there is further variability in learning the first transfer of the instrumental task from the training odor set to a second odor set (Frederick et al. 2017). Thus, to remove the confound of instrumental learning from evaluation of perceptual transfer from retronasal to orthonasal tasks, all rats were pretrained to 2 orthonasal training odor sets that were different from the odorants used in the testing conditions (Table 1) and learned to perform them to at least 80% correct. Once rats learned the orthonasal odor discrimination task, they were then moved to the first round of 2-day retronasal conditioning.

Go/No-Go orthonasal odor discrimination task

Water-restricted rats were trained to discriminate a pair of odorants delivered orthonasally: the CS+ odorant in an airstream predicted delivery of a sweet sucrose solution upon continued licking, and the CS– odorant predicted a bitter tasting quinine-hydrochloride solution with continued licking (Figure 1d). A light at the back of the operant box was illuminated to signify the start of a trial, and the door to the odor port opened after 1 s. An entry attempt to the odor port activated odorized air delivery, and the odor stayed on until the rat retracted from the port. After the onset of odor delivery, the rat could wait for a trial to end in 5 s or reach forward to lick the spout twice to initiate delivery of 40 µL 0.005 M quinine–HCl solution or 40 µL 0.1 M sucrose solution depending on the orthonasal odorant. The light was extinguished 2 s after the reinforcement occurred, and the odor port door

Table 1. Six odor sets were used in the experiment

Phase	Odor 1			Odor 2		
	Odorant	CAS number	Theoretical vapor pressure (kPa, 25 °C)	Odorant	CAS number	Theoretical vapor pressure (kPa, 25 °C)
Pre-training	1,8-Cineole	470-82-6	0.220	Limonene	138-86-3	0.206
Pre-training	Anisole	100-66-3	0.566	Ethyl 2-methylbutyrate	7452-79-1	1.048
High-volatility RPE	2-Hexanone	591-78-6	1.778	Methyl valerate	624-24-8	1.47
High-volatility novel	Octane	111-65-9	1.874	Propyl Propionate	106-36-5	1.902
Low-volatility RPE	Methyl Benzoate	93-58-3	0.051	Amyl Acetate	628-63-7	0.524
Low-volatility novel	Citronellal	106-63-0	0.033	3-Heptanol	589-82-2	0.043

Odorants are matched by vapor pressure and are counterbalanced when assigned to CS+ or CS– during the experiments. Values retrieved from http://pubchem.ncbi.nlm.nih.gov/ or manufacturer websites. Retronasal odorants are used at 0.01% v/v with water. Orthonasal odorants are saturated vapor diluted at approximately 15% with air (Frederick et al. 2017) and delivered at a flow rate of (1.5) L/min.

closed slowly to allow the animal to withdraw from the port to await the next trial. If no nose poke in the odor port was detected, the door closed 10 s after opening. The lick spout was rinsed by a stream of water followed by a vacuum epoch to remove any liquid residue between trials. Each session consisted of 75 CS+ and 75 CS– trials randomly interleaved.

Retronasal conditioning and orthonasal testing

Retronasal trials (Figure 1c) had a similar structure to trials in the orthonasal discrimination task, but clean air was delivered upon nose poke entry to the odor port. One of the 2 odorized aqueous reinforcers, 0.1 M sucrose mixed with a 0.01% CS+ odorant or 0.005 M quinine mixed with 0.01% CS– odorant, was presented with 50% probability once the rat licked twice on the lick spout after the door opened. Odorants volatilized in the air were removed continuously by a concentric vacuum to avoid orthonasal detection (Rebello et al. 2015). All rats were given the opportunity to perform up to 150 trials and all were conditioned for more than 100 trials per session, once per day for 2 days, after which they were tested in the orthonasal paradigm for 2 days. Half of the rats were tested in the orthonasal condition on the RPE odorants, which had been presented in the previous retronasal conditioning sessions, and the other half were tested on a novel odor set matched for volatility in the control condition. Following orthonasal testing, we repeated retronasal conditioning on the same RPE odors for 1 day and then tested the rats on the other orthonasal condition (novel or RPE). The whole process was repeated for the odor sets of different vapor pressure. The testing conditions were within subjects. The testing order and contingency were counterbalanced across subjects.

E2: Retronasal odor discrimination task

E2 is a proof-of-concept study aiming to show that the results from E1 are based on rats' capability to detect and discriminate odorants when presented retronasally (Figure 1e). In these retronasal odor discrimination trials, rats performed a nose poke in the port and licked the spout twice to receive an odorized solution (CS+ or CS–). Any residual odor solution was sucked away by vacuum 3 s after delivery, and rats could respond by either continued licking or withdrawing from the port within 3 s before a trial ended. A correct response was to keep licking the spout for sucrose in response to CS+ or to withdraw from the port in response to CS–. The lick spout was automatically rinsed, and the water removed with the vacuum between trials. The order of test odor sets was counterbalanced for the 4 rats used in E2.

Odorants and tastants

Six pairs of monomolecular odorants (Table 1) were used in E1. Each pair of odorants was matched for theoretical vapor pressure, a measure of volatility or airborne strength, with one odor (CS+) associated with 0.1 M sucrose solution and the other odor (CS–) associated with 0.005 M quinine–HCl solution. To avoid synergetic effects between specific odorants and tastants, odor–taste pairs were counterbalanced across subjects, meaning an odorant was CS+ for half of the rats and CS– for the other half of the rats. During the retronasal conditioning phase, the odorants were mixed with the tastants in water at 0.01% v/v concentration. Four odorants were previously reported to have no taste at the concentration used in the experiment (Blankenship et al. 2019) and therefore were selected to be the 2 RPE odor sets. Hexanone and methyl valerate were the high-vapor-pressure RPE odor set; amyl acetate and methyl benzoate were the low vapor pressure RPE odor set. Although amyl acetate is not normally considered low volatility, given that we only had 4 tasteless odorants, amyl acetate was paired with methyl benzoate in the low-volatility odor set because it is relatively lower in volatility compared with hexanone and methyl valerate. The same odorants were then tested in the orthonasal discrimination tasks in the RPE condition. Novel odorants, including one pair with high vapor pressure (octane, propyl propionate) and one with low vapor pressure (3-heptanol, citronellal), were chosen as control odor sets that rats never experienced before.

Because Experiment 2 was a retronasal odor discrimination task to show that the retronasal odorants at the concentration used in E1 were detectable and discriminable, only the odorants used for retronasal exposure in E1 were used in E2. Different from sniffing the odor in the orthonasal part of E1, rats sampled the odor by licking the spout for a ~40-µL odorized tasteless solution prior to delivery of the odorless taste solution (Gautam and Verhagen 2012).

Statistical analysis

Latency to initiate licking after entry to the odor port and licks made in a trial were extracted from all trials for analysis. The average latency of CS+ and CS– trials for each session for all rats was calculated, and paired-sample *t*-tests were used to compare the mean latency for CS+ and CS– to examine whether rats were capable of discriminating 2 odorants. Performance was measured by the sum of rewarded CS+

trials and unreinforced CS– trials divided by the total attempted trials. We estimated learning rate from the number of trials needed for rats to respond to CS+ and CS– differentially. With a moving block of 5 trials with one trial step increments, a *t*-test was used to compare the latency for CS+ and CS– trials until a significant difference between CS+ latency and CS– latency ($\alpha < 0.05$) was reached for each session. Three-way repeated-measures ANOVAs were used to analyze the effects of retronasal pre-exposure, odor volatility, and test day on session performance and learning rate. Statistical analyses were performed in MATLAB, using functions *anovan* and *multicomp* for post hoc multiple comparison tests with Bonferroni correction.

Results

In the first experiment (E1), we tested whether an odor association formed retronasally could transfer to orthonasal discrimination ability tested via performance and speed of learning. Rats first drank odorized taste solutions, presumed to form a retronasal odor association with the tastant, and then we tested them on the orthonasal discrimination task with the same or different odorants used in the retronasal condition. In the second experiment (E2), the odor and taste solutions were separated during retronasal conditioning to show that rats were able to detect and discriminate the retronasal odorants at the concentration we used in E1.

Response latency and licking behaviors

Rats show different response latencies and licking behaviors to CS+ and CS– in the orthonasal discrimination, which the rats learned (i.e., reached at least 80% correct) before starting the main experiment. They discriminated CS+ and CS– odorants and preferred the sucrose solution over the quinine solution. Figure 2 demonstrates typical behavior during the retronasal conditioning and the orthonasal discrimination. Latency to lick the spout diverged as a rat learned to discriminate odorants in the orthonasal task (Figure 2a, average latency to lick for the demonstrated training session was CS+: 0.62 ± 0.33 s; CS–: 4.2 ± 1.6 s). A 5-s latency means that the rat refrained from licking the spout when the CS– was presented and waited until the end of the trial. To confirm that rats prefer sucrose to quinine solution, the average number of licks for sucrose is significantly higher compared with that for quinine once the reinforcer is delivered (Figure 2b, sucrose: 21.13 ± 4.3 licks; quinine: 6.5 ± 4.5 licks). Corresponding to trials with the 5-s latency, the lick count was zero in Figure 2b when a rat did not respond to the CS–.

The latency to lick in the retronasal conditioning session was not significantly different between CS+ and CS– trials (Figure 2c, average for CS+ response: 0.61 ± 0.58 s; CS–: 0.59 ± 0.35 s), which is consistent with an absence of external cues by which the rats could predict the reinforcer. The lack of differential response for CS+ and CS– at odor sampling was observed for each retronasal session individually when comparing the mean latency to lick for CS+ and CS– in each session (unpaired *t*-tests, all $P > 0.05$). In the retronasal task, once the rats started licking the odor–taste solution, they did show a preference for the CS+ odorized sucrose solution compared with the CS– odorized quinine (Figure 2d, sucrose: 20.9 ± 5.4 lick; quinine: 5.9 ± 2.1 lick). Rats prefer sucrose to quinine solution regardless of the presence of odorants.

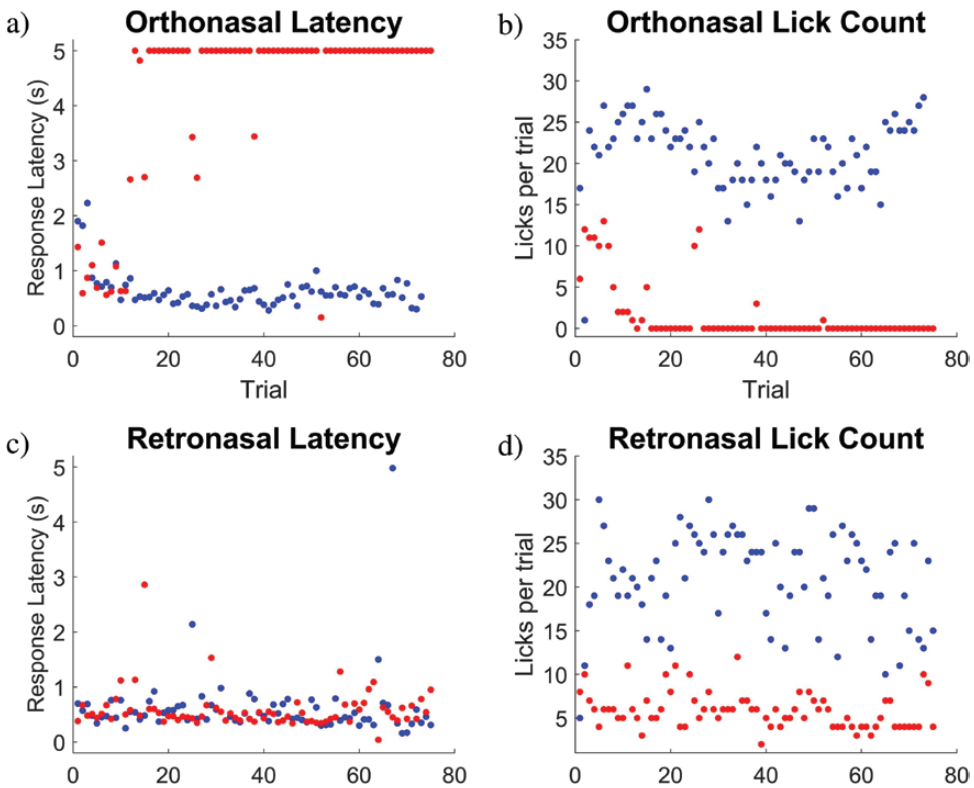

Figure 2. Latency and lick count of retronasal and orthonasal sample sessions. For an orthonasal session, the rat detected the airborne odorant with (**a**) different response latencies for CS+ (blue) and CS– (red), (**b**) licked for the reinforcer sucrose with CS+ or withheld from licking for CS–. Once he received the solution, the number of licks was higher for CS+. For a retronasal pre-exposure session, the rat showed (**c**) no response difference in approaching the lick spout for CS+ versus CS–, but (**d**) the number of licks was higher for sucrose once he received the odor–taste solution.

Effect of retronasal pre-exposure on orthonasal discrimination learning

To analyze the transferability of retronasal experience to orthonasal task performance, we looked at overall performance and learning rate of the orthonasal discrimination using a 2 × 2 × 2 (pre-exposure × volatility × test day) ANOVA. In 2 sessions, 2 rats never reached a significant level of difference between CS+ and CS– before the end of the session. We excluded those sessions because the learning rate could not be estimated, and it suggested that the rats were not engaged in the task due to fatigue or other reasons. Both of these sessions occurred on Day 1 (one low-volatility RPE odor pair and one high-volatility novel odor pair), and to produce an even number of sessions from Day 1 and Day 2, the Day 2 sessions from the same rat and condition were also removed. In total, 4 of the 192 sessions were removed.

For overall performance, the second-day performance was significantly better than the first day performance as rats progressed in the learning process (Day 1 performance: 85.6 ± 1.4%; Day 2 performance: 92.0 ± 1.1%; $F(1,123) = 18.9$, $P < 0.001$, $\eta^2 = 0.16$). We also found a main effect of the odor volatility on performance (high-volatility performance: 91.2 ± 1.0%; low-volatility performance: 86.5 ± 1.6%; $F(1,123) = 6.31$, $P = 0.013$, $\eta^2 = 0.05$), confirming that stronger odors are easier to discriminate. We did not observe a significant main effect of the pre-exposure odorants or interaction between the independent variables in performance attained (Figure 3a).

When examining learning rate (Figure 3b), we estimated the number of trials needed to reach significant response latency differences between odorants in the orthonasal session and found significant main effects of volatility ($F(1,123) = 6.68$, $P = 0.011$, $\eta^2 = 0.06$) and testing day ($F(1,123) = 11.95$, $P < 0.001$, $\eta^2 = 0.10$), indicating that more volatile odorants are learned faster (high volatility 12.4 ± 2.7 trials; low volatility 15.9 ± 4.1 trials) and significant discrimination occurs earlier on Day 2 (9.1 ± 1.4 trials) than Day 1 (14.8 ± 1.0 trials). Retronasal pre-exposure was not significant as a main effect when comparing the learning rate between the RPE odorants and novel odorants, but we observed a significant interaction between RPE and vapor pressure ($F(1,123) = 4.21$, $P = 0.043$, $\eta^2 = 0.036$). We deconstructed the interaction with a post hoc multiple pairwise comparison test with Bonferroni correction of the significance level. The results showed that the animals on average learned to discriminate the high-volatility RPE CS+ and CS– odorants faster than the low-volatility RPE odorants (high-volatility discrimination learned in 7.8 ± 3.8 trials; low volatility 15.4 ± 12.1 trials, $P = 0.008$). The other pairwise comparisons showed no significant differences; learning rate for the novel high-volatility odorants and low-volatility odorants was 11.7 ± 10.6 trials and 12.6 ± 9.9 trials, respectively ($P = 1$ with Bonferroni correction).

If retronasal and orthonasal olfaction generate similar percepts, and retronasal odor–taste associations can be maintained during orthonasal sampling, we would predict faster learning of orthonasal associations or better performance when odorants presented in the orthonasal discrimination tests are retronasally pre-exposed compared with the novel ones. Although the performance was not significantly different, the interaction in the learning rate shows that the effect of retronasal pre-exposure is dependent on volatility in that stronger odorants are more likely to transfer quickly from the retronasal to the orthonasal route and generate a similar percept.

Rats can detect and discriminate retronasal odors

The design of E1 was based on 2 assumptions that the odorants were detectable in the solutions and that rats retain the memory of the RPE odor for 2 days while we tested them on the orthonasal task. We performed a follow-up experiment E2 with 4 animals to test these assumptions. Rats first sampled an odorized solution in plain water and could continue licking for the following associated taste reinforcer. For the first set of odorants, the rats took on average 5.25 ± 1.89 days to perform the task above 75% (Figure 4a). Once they mastered the first task (correct > 85%), they were transferred to the second retronasal odor set. The rats were able to perform the task at above 68% the first day on the new sets and maintained a correct rate above 65% for the 3 days of testing. Therefore, rats were able to detect and discriminate the retronasal odorants at the concentration used in E1. Additional rats tested for another study

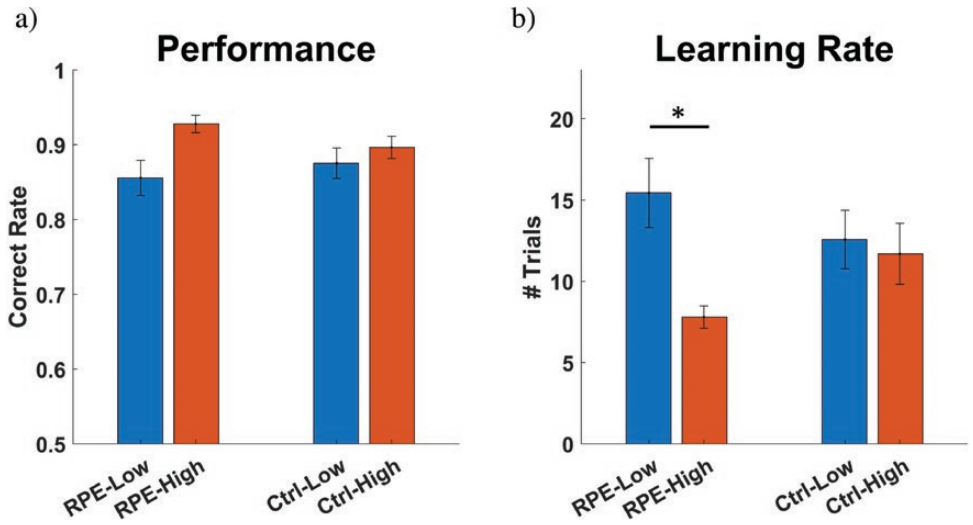

Figure 3. Overall performance (**a**) and learning rate (**b**) across conditions for Experiment 1. (a) There was a main effect on the odorant volatility (high orange, low blue; $P = 0.01$), but not on the retronasal pre-exposure on the overall performance ($P = 0.9$). (b) The trials needed to learn the odorants in the orthonasal task are significantly fewer for retronasal pre-exposed high-volatility odorants. Asterisk indicates significant learning rate differences between the retro-low and retro-high odorants.

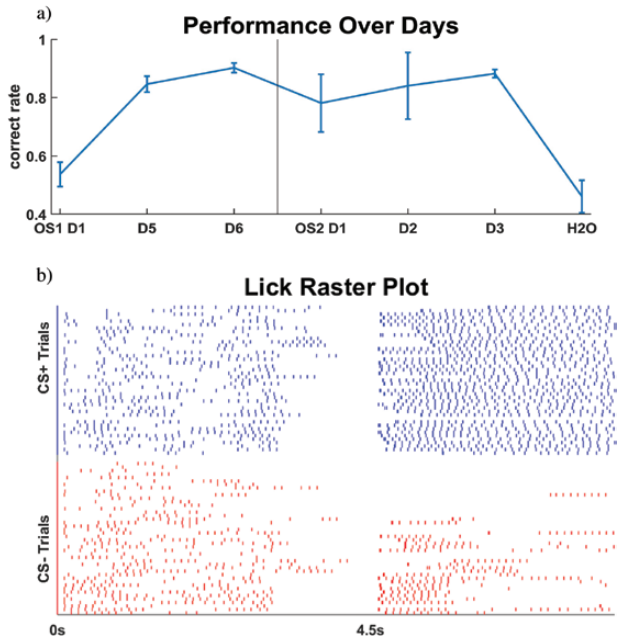

Figure 4. (**a**) Performance over days for E2 in which rats ($n = 4$) were trained in a retronasal task to discriminate the odorants in solution at the concentration used in E1. Performance started at approximately 50% chance level, reached 90% at Day 6, and maintained at 80% when the rats were transferred to the other odor set. When odorized solutions were replaced with water, the performance dropped back to chance level. (**b**) Example of a lick raster plot sorted by trial types over time (blue: CS+; red: CS−; earlier trials on the bottom). Note that the rat ceases producing the second lick bout after smelling the CS− odorant retronasally after around 20 trials of CS−.

showed that they too learned the odor-taste association quite reliably within the first 2 sessions (data not shown). The instrumental behavior to continue to lick the lick spout to receive more water and the reinforcer, which was not required for the odor–taste mixtures in E1 retronasal exposure, may be hard to master and required more than 2 days of practice for the first odor set. However, because all rats learned the discrimination on the first day of the second odor set (order of odor sets counterbalanced order across rats), we can conclude that associations are formed quickly even if the instrumental learning used in E2 takes a few days initially. To exclude possible contextual cues other than retronasal odorants, the odorized solutions were replaced by plain water and performance decreased to 46.1 ± 5.6%, which is not different from chance.

One might argue that the task in E2 is not identical to the retronasal conditioning phase in E1, where the tastant is present with odorant, and the rats may not be able to retain the odor's hedonic valence separately when an odorant and a tastant are mixed in the same solution. We can address this through a fortuitous experimental mistake; we first put 2 previously RPE-conditioned rats in the pilot E2 study for the experiment 2 months after their previous retronasal conditioning. When the tastant was no longer mixed in the solution but was separated as the reinforcer, on the first few days of training, the rats showed that they retained odor associations learned previously. They licked significantly less in response to a pure odorized solution to obtain the reinforcer, if the odorant had been previously mixed with the bitter taste, and they continued licking if the odor had been previously mixed with a sweet taste (data not shown). Overall, E2 showed that rats can not only detect and discriminate the odorants in the solution, but they can also remember the odor information when testing in the subsequent tasks at least 2 months later.

Discussion

Many studies have compared retronasal and orthonasal olfactory routes looking at neural responses and sampling behaviors, but we still understand relatively little regarding whether and how learning between 2 different routes interact perceptually in freely behaving animals. In this study, we examined whether the perceptual quality is similar in the 2 routes using a retronasal conditioning transfer paradigm. If a chemical evokes similar percepts via both routes, animals should perform better or learn faster to discriminate orthonasally 2 odorants previously learned retronasally because the retronasal conditioning has already established an odor–taste association. Our study has mixed outcomes; compared with the novel odorants, the RPE odorants did not show main effects of significantly better performance or learning rate, which supports the theory that there exists a duality of olfactory perception via the 2 olfactory routes (Rozin 1982). Nevertheless, we observed an interaction between retronasal pre-exposure and odorant volatility, that more volatile RPE odorants are learned faster compared with the less volatile RPE odorants, suggesting that retronasal pre-exposure affects subsequent orthonasal learning in a volatility-dependent manner. The results suggest that transference from retronasal to orthonasal experience is possible depending on odorant volatility, which implies that the 2 routes may share perceptual quality. The dual percept theory predicts that pre-exposure to retronasal experience with odors would not affect orthonasal learning, and these results cast doubt on that theory.

A recent study by Blankenship et al. (2019) argued that there is no apparent transfer of odor percept from the retronasal to the orthonasal route. In that study, rats were conditioned with 2 odorized solutions delivered passively via intraoral cannula (i.e., retronasal stimulation), with one odorant followed by oral infusion of a sucrose solution and the other reinforced by plain water. After the conditioning, rats preferentially nose-poked to an odor port that was associated with retronasal odorants paired with sucrose. The conditioned retronasal odor preference, measured by increased nose poking to the odor port associated with the CS+ odorant delivered orthonasally, failed to transfer the preference to airborne odorants, suggesting that retronasal and orthonasal odorants are perceptually different. In our Experiment 1, performance estimated for the whole session of 150 trials was not significantly different between retronasally pre-exposed and novel odorants, and this negative result is consistent with the hypothesis that the 2 pathways produce different percepts. However, the lack of a performance difference may be attributed to a ceiling effect. The rats in our study were pre-trained in the orthonasal discrimination task on different odors, and after learning to transfer, the learned rule to new odor sets performance is high and robust to the subsequent odor sets (Frederick et al. 2017). Therefore, the rats performed at high performance levels overall (88 ± 11%), and learning rate instead of performance may be a better indication of perceptual transfer for the task. Moreover, we used a different training paradigm from that in the study by Katz et al. (2018); in our study, licking to sample the retronasal odorants is an instrumental response rather than passive oral infusion. The sampling procedure is more similar to naturalistic flavor learning, perhaps engaging the animals to better process the odor identity, and the licking may deliver the retronasal stimulus more efficiently to the retronasal pathway. In Experiment 1, we also positively reinforced one odorant with sucrose and negatively

reinforced the other with quinine, which may have promoted avoidance behaviors and lead to a more salient effect.

One parsimonious explanation of our results may be that the different learning rates we observed for the RPE odors sets are odor-specific for each tested orthonasal pair. Although we have not observed significant odorant differences in learning rate in olfactory discrimination tasks used in the lab unless the odorants are very similar to each other (Frederick et al. 2017), we cannot rule out this explanation due to the limit in choices of tasteless odorants used in the study. In this study, the odor-taste association within an odorant pair as well as the order of testing conditions were counterbalanced for all animals, such that the presentation order of odorants and the synergic effect of odor-taste association should have minimal odor-specific learning effects on the results. Our study showed that specific odorants or the physicochemical properties (e.g., volatility) of odorants and the behavioral training and testing paradigm may play significant roles in retronasal/orthonasal olfactory learning, and these effects should be accounted for in future studies.

Odor volatility affects perceptual threshold

The same chemical may generate a similar percept via 2 routes as a combined result of the physical attributes of odorants and the 2 olfactory routes. We focused on the effect of theoretical vapor pressure as a measure of volatility and observed that the high-vapor-pressure odorants were easier to discriminate orthonasally and were learned faster when they had been retronasally pre-exposed with tastants. One factor in the interaction we observed between pre-exposure status and volatility is that the 2 olfactory routes show physical differences in humidity, temperature, and airflow, which may strongly interact with solubility. This poses a conundrum regarding systematic characterization of a chemical environment when odorants enter the nasal epithelium. To address the limitation, Scott et al. (2014) simulated electro-olfactogram (EOG) activation at the nasal epithelium with thermodynamic modeling and showed that EOG amplitude follows a curvilinear shape as a function of solubility for both orthonasal and retronasal routes, but the retronasal route has a lower amplitude due to concentration differences and the limitation of flow rate. Monomolecular odorants within the range of medium to high mucosal solubility (estimated by air/mucus partition coefficient) elicited relatively high intensity among ~300 odorants simulated, and the more volatile odorants we chose fall within this range of solubility. It is possible that there exists a threshold of some physical attribute such as solubility, volatility and/or concentration of odorants for perceptual similarity across the 2 routes because learning transferred in our paradigm only for the high-volatility odorants. This perceptual threshold may be different from the detection threshold and is supported by the weaker but similar glomerular activation patterns for retronasal versus orthonasal stimuli (Gautam and Verhagen 2012). In the second experiment, we demonstrated rats' ability to detect and discriminate the odorants at the concentration used in E1 for both high- and low-volatility odorants, and the disparity in learning speed between the high and low pre-exposed odorant is not caused by the rats failing to detect low-volatility odorants.

Due to the limited choice of known tasteless odorants at the time that we planned this experiment, hexanone and methyl valerate were the only pair of high-volatility odorants that rats experienced retronasally, and therefore the faster learning rate could be because these 2 odorants are more easily distinguished orthonasally. We also considered the possibility of retronasal odorant leak if the vacuum is not sufficient or if odorized liquid remains on the rats' whiskers so that they smelled the odorants orthonasally. These both would predict increased learning or performance for both low- and high-volatility odorants instead of the interaction observed in our study.

Neural explanations for shared percepts between routes

Our study showed that an odor percept may transfer from the retronasal route to the orthonasal route if the odor stimulus is strong enough (highly volatile or highly soluble). This may mean that the transfer depends on concentration, and there is ample evidence that neural circuitry in the olfactory bulb can produce concentration invariance in the physiological response. Olfactory information, by its nature, depends on concentrations of odorants that may fluctuate within a sniff cycle, over a breathing period or between 2 olfactory routes, but the odor quality is maintained. Imaging studies on the periphery show the variance in input from olfactory sensory neurons in the nasal epithelium is normalized and sequentially gives rise to a more consistent output amplitude and spatial distribution in the mitral and tufted cells (Storace and Cohen 2017). Cortical or higher order neural responses in mammals and insects, which arguably represent odor identity, have a more reliable odor-specific firing pattern regardless of various concentration levels of an odorant (Stopfer et al. 2003; Wilson and Sullivan 2011; Cleland et al. 2012; Bolding and Franks 2017). Spatial encoding of odor perceptual quality may serve as another argument for similar percepts across routes. Prior studies have shown that glomerular activation patterns may represent a "map" for odor identity (Johnson and Leon 2000; Meister and Bonhoeffer 2001; Wachowiak and Cohen 2001), and overlapping of glomerular activation can predict perceptual quality overlap (Linster et al. 2001). Odorants from the 2 routes do evoke a similar glomerular ensemble once the odor information activates glomeruli as imaged on the dorsal OB (Gautam and Verhagen 2012). The source of a difference in perceptual quality between retronasal and orthonasal routes is thus likely to arise somewhere besides the glomerular activation pattern. The same study also showed that retronasal odorants arrive at the nasal epithelium more slowly compared with orthonasal odorants to initiate a response cascade. However, the temporal encoding of odor identity is relatively understudied for the 2 routes once an odorant binds to the olfactory receptors. Future experiments may include looking at the oscillatory events in the OB and in higher cortical and subcortical regions in the olfactory system to investigate local and coordination of neural activities.

It is important to understand how animals are able to generate consistent perception yet maintain distinctive information about the source of odors when odorants enter from 2 different routes in flavor acquisition and odor learning. It is equally important to understand how multimodal effects such as odor and oral or nasal sensation may fundamentally alter perceptual experience. Odorant-evoked behaviors and engagement of the neural systems may help in unraveling perceptual convergence and divergence of 2 olfactory inputs identical in chemical profile. In this study, we showed that learning transfer from retronasal to orthonasal perception is possible and depends on the odorant properties, suggesting that there can be a shared percept across the routes, contradicting the duality of smell theory (Rozin 1982).

Conflict of interests

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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