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Olfactory discrimination ability of CD-1 mice for a large array of enantiomers

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Abstract

With use of a conditioning paradigm, the ability of eight CD-1 mice to distinguish between 15 enantiomeric odor pairs was investigated. The results demonstrate a) that CD-1 mice are capable of discriminating between all odor pairs tested, b) that the enantiomeric odor pairs clearly differed in their degree of discriminability and thus in their perceptual similarity, and c) that pre-training with the rewarded stimuli led to improved initial but not terminal or overall performance. A comparison between the proportion of discriminated enantiomeric odor pairs of the CD-1 mice and those of other species tested in earlier studies on the same discrimination tasks (or on subsets thereof) shows a significant positive correlation between discrimination performance and the number of functional olfactory receptor genes. These findings provide the first evidence of a highly developed ability of CD-1 mice to discriminate between an array of non-pheromonal chiral odorants. Further, they suggest that a species' olfactory discrimination capabilities for these odorants may be correlated with its number of functional olfactory receptor genes. The data presented here may provide useful information for the interpretation of findings from electrophysiological or imaging studies in the mouse and the elucidation of odor structure-activity relationships.

Introduction

The neural mechanisms underlying the ability of many species to recognize and discriminate between a vast number of structurally diverse odorants is arguably one of the central topics in olfactory research. A great deal of the current knowledge about the coding of olfactory information, and of odor quality coding in particular, has been obtained using the mouse as a model species (e.g. Katada et al., 2005; Shimshek et al., 2005; Wachowiak et al., 2005; Zou et al., 2005). With the notable exception of body-borne odors (e.g. Beauchamp and Yamazaki, 2003; Schaefer et al., 2002; Wysocki et al., 2004; Yamazaki et al., 1994), however, surprisingly few studies have so far assessed olfactory discrimination performance in the mouse at the organismal level. This is all the more surprising given the importance of basic data on discriminative abilities with monomolecular odorants for the interpretation of findings from electrophysiological or imaging studies (e.g. Johnson et al., 2004; Xu et al., 2003, 2005) and the elucidation of odor structure-activity relationships (Mori et al., 2006). Enantiomers are pairs of molecules with mirror image structures that exhibit identical chemical and physical properties except for their optical activity, that is, rotation of polarized electromagnetic waves. They are particularly useful for assessing how molecular

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structure is encoded by the olfactory system, as a basis for discriminable odor qualities, because perceptual differences between enantiomers cannot be due to properties such as differing diffusion rates in the mucus covering the olfactory sensory epithelium or differing air/mucus partition coefficients (Hahn et al., 1994), but must originate from chiral selectivity at the peripheral receptor level (Rossiter, 1996). Therefore, the systematic assessment of the discriminability of enantiomeric odor pairs may contribute to our understanding of odor quality perception and coding.

In order to begin to provide the needed data, it was the aim of the present study to assess the olfactory discrimination performance of CD-1 mice for 15 pairs of enantiomers. Substances were chosen on the basis of earlier studies that used the same stimuli (or subsets thereof) with other species such as squirrel monkeys (Laska et al., 1999, 2005.), pigtail macaques (Laska et al., 2005.), honey bees (Laska and Galizia, 2001), rats (Linster et al., 2002; Rubin and Katz, 2001), mole rats (Heth et al., 1992) and also human subjects which were only able to distinguish between 5 of the 15 enantiomeric odor pairs (Laska and Teubner, 1999; Laska, 2004). This allowed us to additionally address the question whether a species' olfactory discrimination capabilities with enantiomers may be correlated with its number of functional OR genes.

Material and methods

Animals

Testing was carried out using eight male CD-1 mice (*Mus musculus*). The rationale for choosing this outbred strain of mice was to use animals with a variable genetic background that is more similar to wild-type mice than that of inbred strains. Furthermore, data on olfactory detection thresholds for the enantiomers of carvone and limonene (Joshi et al., in press) were obtained in an earlier study using the same mouse strain. Maintenance of the animals has been described in detail elsewhere (Laska et al., 2006). The mice were 150-170 days old at the beginning of the study. During the experiments, the animals were kept on a water deprivation schedule of 1.5 ml of water per day. The experiments reported here comply with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication no. 86-23, revised 1985) and were performed according to a protocol approved by the Yale University Institutional Animal Care and Use Committee.

Stimuli

A set of 30 odorants comprising 15 pairs of enantiomers was used (Table 1). The rationale for choosing these substances was to use stimulus pairs for which comparative data on discrimination performance from other species are at hand (Heth et al., 1992; Laska, 2004; Laska and Galizia, 2001; Laska and Teubner, 1999; Laska et al., 1999a, 2005; Linster et al., 2002; Rubin and Katz, 2001). All substances were obtained from Sigma-Aldrich (St. Louis, MO) and Fluka (Seelze, Germany) and had a nominal purity of at least 99%. They were diluted using odorless diethyl phthalate (Sigma-Aldrich) as the solvent. All odorants were presented at a gas phase concentration of 1 ppm (parts per million) as calculated using the formulae provided by Weast (1987). This concentration excludes the possibility that the nasal trigeminal system might have contributed to the discrimination of stimuli.

Behavioral test

Olfactory discrimination performance of the mice was assessed using an automated olfactometer (Knosys, Tampa, FL). Animals were trained using standard operant conditioning procedures (Bodyak and Slotnick, 1999) to insert their snout into the odor sampling port of a test chamber. This triggered the 2 s presentation of either an odorant used as the rewarded stimulus (S+) or a different odorant used as the unrewarded stimulus (S-).

Licking at a steel tube providing 2.5 μ l of water reinforcement in response to presentation of the S+ served as the operant response. Six blocks of 20 such trials (totalling 60 S+ and 60 S- trials in pseudorandomized order) using a given stimulus pair were conducted per animal and task. Olfactory discrimination performance was determined by testing the animals' ability to distinguish between one optical isomer of a given substance used as S+, and the other optical isomer of the same substance used as S-. In order to assess the effect of pre-training with one of the optical isomers of a given enantiomeric odor pair on discrimination performance, four of the eight animals received two sessions of 120 trials each using the assigned optical isomer as S+ and anethole as S-, followed by two sessions of 120 trials each using the same S+ and cineol as S- on the days prior to the critical test. The four other animals did not receive any kind of pre-training with any of the optical isomers but were presented with an easy control task (anethole as S+ versus cineol as S-) on the day prior to the critical test. Within each of the two groups, two animals were assigned the (+)-form of a given substance as S+, and the other two animals were assigned the (-)-form as S+.

The order of presentation of the stimuli followed the sequence given in table 1. To test whether the order of presentation affected differences in discriminability, two odor pairs (the carvones and the limonenes) were re-tested at the end of the study and their results compared to those of initial testing.

Data analysis

For each individual animal, the percentage of correct choices from 120 decisions per stimulus pair was calculated. Correct choices consisted both of licking in response to presentation of the S+ and not licking in response to the S-, and errors consisted of animals showing the reverse pattern of operant responses. Additionally, the percentage of correct choices in the first block of 20 trials per task (comprising 10 S+ and 10 S- trials in pseudorandomized order), and in correct rejections of the S- in the first block of 20 trials per task was analyzed. Significance levels were determined by calculating binomial z-scores corrected for continuity from the number of correct and false responses for each individual and condition. Comparisons across tasks were made using Friedman's two-way analysis of variance. When ANOVA detected differences between tasks, this was then followed by pairwise Wilcoxon's signed-rank tests for related samples to evaluate which tasks were responsible. Comparisons of performance between groups of animals were made using the Mann-Whitney U-test for independent samples. Correlations between the across-task patterns of performance of the mice and other species were evaluated using Spearman's rank correlation coefficient and tested for significance by computing t values. All tests were two-tailed, and the alpha level was set at 0.05. Bonferroni corrections for multiple tests were performed where appropriate.

Results

Figure 1 summarizes the mean performance of the eight mice in discriminating between the 15 enantiomeric odor pairs. When considering the mean percentage of correct decisions across the six blocks of 20 trials performed per animal and task (Fig. 1, circles), the mice performed significantly above chance in all tasks (binomial test, $p < 0.01$). Interindividual variability with a given task was low as can be inferred from the small SEs, and with only one exception (one animal failed in the task (+)- versus (-)-rose oxide) all eight animals scored significantly above chance level in all tasks. Mean scores ranged from 77.6 % for the discrimination of the enantiomers of rose oxide to 95.6 % for the discrimination of the optical isomers of dihydrocarvyl acetate. Accordingly, ANOVA detected significant differences in the group's performance between tasks (Friedman, $p < 0.001$) and subsequent pairwise tests revealed that the enantiomers of rose oxide, perilla alcohol, limonene, isopulegol, and 2-butanol were significantly more difficult to discriminate compared to most

of the other odor pairs, and that the optical isomers of dihydrocarvyl acetate were significantly easier to discriminate than all other odor pairs except menthol (Wilcoxon, $p < 0.05$). Differences in performance between tasks were even more pronounced when considering only the percentage of correct decisions in the first block of 20 trials performed per animal and task which ranged from 55.0 % with rose oxide to 80.0 % with dihydrocarvyl acetate (Fig. 1, squares), or the percentage of correct rejections of the unrewarded stimulus (S−) in the first block of 20 trials which ranged from 15.0 % with rose oxide to 61.3 % with menthol (Fig. 1, triangles). The across-task patterns of performance correlated significantly between all three measures of performance (Spearman, $r_s \geq 0.92$, $p < 0.001$ with all three comparisons).

The mean percentage of correct decisions across the 15 discrimination tasks of the four animals that received pre-training (88.4 ± 7.6 %) and that of the four animals that did not receive pre-training (87.5 ± 5.7 %) did not differ significantly from each other (Mann-Whitney, $p > 0.05$). However, figure 2 shows that the pre-trained mice scored significantly better in blocks 1 and 2 than the animals without pre-training (Mann-Whitney, $p < 0.01$). Conversely, the animals without pre-training displayed significantly higher mean scores of correct decisions compared to the pre-trained mice in blocks 5 and 6 (Mann-Whitney, $p < 0.01$).

Two of the tasks (the discriminations between the two carvones and the two limonenes, respectively) were re-tested after completion of the 15 discrimination tasks. The mean percentages of correct decisions across the six blocks of 20 trials performed per animal and task in the re-tests (94.5 % for the discrimination of the carvones, and 83.3 % for the discrimination of the limonenes) did not differ significantly from the percentages in the initial tests (Wilcoxon, $p > 0.05$). From this, and from the fact that carvone had been tested as the very first odor pair and limonene as the fourth odor pair in the initial series (see Table 1) we conclude that test order did not affect the observed differences in performance between tasks. In a control test using the solvent both as S+ and as S−, all eight animals performed at chance level, suggesting that the observed differences in performance between tasks was based on differences in perceived similarity of the odorants and not on unwanted cues.

Discussion

The results of this study demonstrate that CD-1 mice are capable of discriminating between all 15 enantiomeric odor pairs tested, and that the enantiomeric odor pairs significantly differed in their degree of discriminability and thus in their perceptual similarity. Further, the results show that pre-training with the rewarded stimuli led to improved initial but not terminal or overall performance.

Our finding that CD-1 mice discriminated between all 15 enantiomeric odor pairs tested raises the question whether perceived differences in stimulus intensity rather than stimulus quality might have contributed to the excellent discrimination performance. Although this possibility cannot be ruled out completely, it appears rather unlikely as, firstly, CD-1 mice have been shown to display similar, if not identical, olfactory detection thresholds for the optical isomers of carvone (Joshi et al., 2006); secondly, the ability of mice to learn to discriminate between different concentrations of a stimulus in the conditioning paradigm used here is limited (Slotnick, personal communication); and thirdly, increasing or decreasing the concentration of either the S+ or the S− by a factor of 10 had no significant effect on mean discrimination performance (limonene, initial test : 82.0 %, test with S+ ten-fold higher : 82.9 %, test with S+ ten-fold lower: 80.9 %, test with S− ten-fold higher: 83.5 %, test with S− ten-fold lower: 80.6 %, Wilcoxon, $p > 0.05$ for all comparisons). Therefore,

we believe the discrimination scores found here to reflect the ability of the mice to distinguish between stimulus qualities.

Table 2 compares the performance of the mice to that of other species tested on the same discrimination tasks or on subsets thereof. Human subjects were only able to significantly discriminate between 5 of the 15 enantiomeric odor pairs tested here (Laska and Teuber, 1999; Laska, 2004), and squirrel monkeys distinguished between 6 of the 15 pairs of optical isomers (Laska et al., 1999, 2005). Pigtail macaques were able to score above chance level with 7 out of 9 odor pairs (Laska et al., 2005), and honey bees discriminated the (+)- and (-)-forms with 4 out of 8 of the substances tested here (Laska and Galizia, 2001). Rats and mole rats, in contrast, succeeded in discriminating between all 4, respectively 3, enantiomeric odor pairs tested (Linster et al., 2002; Rubin and Katz, 2001; Heth et al., 1992).

Recent genetic studies have shown that mice have approximately 1,000 different functional genes coding for olfactory receptors (Godfrey et al., 2004; Zhang and Firestein, 2002) whereas squirrel monkeys (≈ 800), pigtail macaques (≈ 700), human subjects (≈ 350) and honey bees (≈ 160) have considerably lower numbers (Gilad et al., 2004; Glusman et al., 2001; Robertson, personal communication). This raises the possibility that the number of OR types expressed in the olfactory epithelium may be correlated with a species' ability to discriminate between structurally related odorants. The finding that rats, another rodent species known to have a large repertoire of $\approx 1,200$ functional OR genes (Quignon et al., 2005), were also able to distinguish between all enantiomeric odor pairs tested with them so far is in line with this idea. Figure 3 shows that indeed a significant positive correlation between the number of functional OR genes and the proportion of discriminated enantiomeric odor pairs can be found based on the data in Table 2 (Spearman, $r_s = 0.81$, $p < 0.05$). It should be mentioned, however, that only a subset of 100 squirrel monkey and macaque OR genes have been analyzed so far and that proportions of 19% and 29% of pseudogenes, respectively, within this subset led Gilad et al. (2004) to conclude that these two primate species might possess the total numbers of functional OR genes mentioned above. Thus, to further corroborate the hypothesis that the number of functional OR genes determines a species' discriminative abilities with olfactory stimuli, future studies should aim at identifying the total number of functional OR genes in a larger variety of species, and testing those species with sets of the same olfactory discrimination tasks.

It is interesting to note that all species listed in Table 2 were capable of distinguishing between the optical isomers of limonene, carvone, dihydrocarvone, dihydrocarveol, and dihydrocarvyl acetate. In contrast, only the mice (and, in the case of 2-butanol, also the rats), but none of the non-rodent species were able to discriminate between perillaaldehyde, limonene oxide, camphor, rose oxide, and 2-butanol. All five enantiomeric odor pairs that were discriminated by all species listed in Table 2 share a functional isopropenyl group at the chiral center. However, perillaaldehyde and limonene oxide which were only distinguished by the mice but not by human subjects, squirrel monkeys and pigtail macaques also share this structural feature suggesting that factors other than the functional group attached to the chiral carbon atom might account for the ability of all species tested so far to discriminate between the same subset of enantiomeric odor pairs. In honey bees a significant positive correlation between discrimination scores for enantiomers and the frequency of occurrence of these compounds in flower odors has been reported (Laska and Galizia, 2001). The authors interpret this finding as an evolutionary adaptation of the honey bee's olfactory system to its chemical environment. Using the same database of floral scent compounds from more than 400 plant taxa (Knudsen et al., 1993) no such correlation was found with the mice tested here (Spearman, $r_s = 0.03$, $p > 0.05$). However, the facts that the mice significantly differed in their discrimination scores with the different enantiomers (see Fig. 1), and that their chemical environment clearly differs from that of the honey bee, leave the

possibility that the frequency of occurrence of substances in the odorous environment of the mouse may correlate with its relative discrimination performance for different odor pairs. Unfortunately, with the exception of body-borne social odors, very little is known about the composition of the chemical environment of the mouse. To test the above-mentioned hypothesis, it is therefore clearly important to get a better idea of the composition and frequency of occurrence of odorants in the natural habitat of this and of other species used as animal models in olfactory research.

Our finding that the enantiomeric odor pairs clearly differed in their degree of discriminability and thus in their perceptual similarity raises the question whether these differences are mirrored by differing degrees of similarity in the neural representations evoked by the stimuli. Linster et al. (2001) demonstrated that stimulation with the enantiomers of carvone led to overlapping but significantly distinct odor maps based on the uptake of [^{14}C]2-deoxyglucose in the rat olfactory bulbs, whereas glomerular activation patterns in response to the enantiomers of limonene were not statistically different from each other. In line with these findings, rats were found to spontaneously discriminate between the optical isomers of carvone but not of limonene although the animals were capable of learning to distinguish between (+)- and (–)-limonene when a differential reinforcement paradigm was employed (Linster et al., 2002). The range of discrimination performance found with the 15 different pairs of enantiomers in the present study offers the possibility to systematically assess possible correlations between perceptual similarity of odorants and their neural representations in future studies using powerful imaging techniques such as fMRI (Xu et al., 2003, 2005).

A final aspect of the present study is our finding that mice that received pre-training with the S+ did not show higher mean scores of discrimination than mice that did not receive pre-training. This appears to be in contrast with several studies that reported experience to facilitate olfactory discrimination in humans (Rabin, 1988; Hudson, 1999) as well as in rats (Fletcher and Wilson, 2002; Wilson and Stevenson, 2003; Mandairon et al., 2006) and mice (Magavi et al., 2005; Kerr and Belluscio, 2006). However, figure 2 illustrates that pre-trained mice indeed displayed significantly higher percentages of correct discriminations in the first two blocks of 20 trials compared to mice without pre-training, suggesting that experience with one of the discriminanda may positively affect initial performance. Our finding that the mice without pre-training, in turn, demonstrated higher scores of correct decisions in the fifth and sixth block of 20 trials compared to the pre-trained animals could be explained by a higher degree of sustained vigilance after initial task acquisition in the mice without pre-training due to the relative novelty of both stimuli (Sutherland and Mackintosh, 1971). The mechanisms thought to underlie experience-dependent increases in olfactory discrimination performance include accelerated glomerular refinement (Kerr and Belluscio, 2006), modifications of granule cell responsiveness in the olfactory bulb (Magavi et al., 2005), and plasticity of odorant receptive fields in the piriform cortex (Fletcher and Wilson, 2002; Wilson and Stevenson, 2003). Future studies could take advantage of the conditioning paradigm used here to elucidate which of these mechanisms may be involved during initial task acquisition, that is, during the first 40 or, possibly even fewer, trials with a given discrimination task.

Taken together, the results of the present study provide the first evidence of a highly developed ability of CD-1 mice to discriminate between an array of non-pheromonal chiral odorants. A comparison between the performance of the mice and those of other species tested on the same tasks suggests that a species' olfactory discrimination capabilities may be correlated with its number of functional OR genes.

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Figure 1. Performance of eight CD-1 mice in discriminating between 15 pairs of enantiomers presented at a gas phase concentration of 1 ppm. Each data point represents the percentage (mean $\pm$ SE) of correct decisions per odor pair a) across the six blocks of 20 trials performed per animal and task (circles), b) in the first block of 20 trials (squares), and c) in correct rejections of the S- in the first block of 20 trials (triangles).

Figure 2.

Performance of CD-1 mice in the six blocks of 20 trials performed per animal and task. Each data point represents the percentage (mean $\pm$ SE) of correct decisions across the 15 discrimination tasks a) of the four animals that received pre-training with the S+ (circles), and b) of the four animals that did not receive pre-training with the S+ (squares). **: $p < 0.01$ ns: not significant (Mann-Whitney).

Figure 3.

Comparison of the proportion of discriminated enantiomeric odor pairs of the CD-1 mice and those of other species tested on the same discrimination tasks (or on subsets thereof) as a function of the number of functional OR genes. The solid line indicates the regression according to the Spearman rank-correlation test.

Table 1

Substances and concentrations used

substance pair	chemical description	liquid phase concentration (g/l)
(+)- vs. (-)- carvone	monocyclic terpene-ketone	90.5
(+)- vs. (-)-dihydrocarvone	monocyclic terpene-ketone	71.5
(+)- vs. (-)- dihydrocarveol	monocyclic terpene-alcohol	76.9
(+)- vs. (-)-limonene	monocyclic terpene-hydrocarbon	13.0
(+)- vs. (-)-2-butanol	aliphatic alcohol	0.7
(+)- vs. (-)-limonene oxide	monocyclic terpene-epoxide	45.5
(+)- vs. (-)- dihydrocarvyl acetate	monocyclic terpene-ester	111.1
(+)- vs. (-)-isopulegol	monocyclic terpene-alcohol	45.5
(+)- vs. (-)-perillaaldehyde	monocyclic terpene-aldehyde	126.6
(+)- vs. (-)-perillaalcohol	monocyclic terpene-alcohol	83.2
(+)- vs. (-)-rose oxide	monocyclic terpene-oxide	90.5
(+)- vs. (-)-fenchone	bicyclic terpene-ketone	23.2
(+)- vs. (-)-camphor	bicyclic terpene-ketone	33.4
(+)- vs. (-)-menthol	monocyclic terpene-alcohol	45.5
(+)- vs. (-)- β -citronellol	acyclic terpene-alcohol	76.9

Table 2

Across-species comparison of discrimination performance with enantiomers

	CD-1 mice	human subjects	squirrel monkeys	pigtail macaques	honey bees	SD/LE rats	mole rats
limonene	+	+	+	+	+	+	
carvone	+	+	+	+	+	+	+
dihydrocarvone	+	+	+	+			
dihydrocarveol	+	+	+	+			
dihydrocarvyl acetate	+	+	+	+			
perillaaldehyde	+	–	–	–			
perillaalcohol	+	–	–	+			
isopulegol	+	–	–	+			
limonene oxide	+	–	–	–			
camphor	+	–	–	–			
fenchone	+	–	+	–	–	+	+
rose oxide	+	–	–	–			
menthol	+	–	–	+	+		
β-citronellol	+	–	–	–	+		+
2-butanol	+	–	–	–	–	+	

Data for human subjects : Laska and Teubner, 1999; Laska, 2004.

Data for squirrel monkeys : Laska et al., 1999, 2005.

Data for pigtail macaques : Laska et al., 2005.

Data for honey bees : Laska and Galizia, 2001.

Data for Sprague Dawley (SD) rats (carvone and limonene) : Linster et al., 2002.

Data for Long Evans (LE) rats (carvone, fenchone, and 2-butanol) : Rubin and Katz, 2001.

Data for mole rats : Heth et al., 1992

⁺ indicates the ability to significantly discriminate between an enantiomeric odor pair.

[–] indicates the failure to significantly discriminate between an enantiomeric odor pair.