The mammalian olfactory system can detect and discriminate an enormous variety of volatile molecules. The first steps of this sensory process are performed by olfactory receptor neurons (ORNs), which interact with distinct odorants and transduce the chemical stimulus into electrical signals. Odorants interact with G-protein-coupled receptors (Buck and Axel, 1991) located in olfactory cilia (Menco et al., 1997), thus triggering the formation of second messengers which, in turn, elicit an influx of cations, including Ca\(^{2+}\) (for reviews, see Breer et al., 1994; Reed, 1994; Shepherd, 1994). Thereby, the increase in Ca\(^{2+}\) levels elicited by odour stimuli mirrors the responsiveness of ORNs, which can be monitored using Ca\(^{2+}\) imaging (Restrepo et al., 1993; Tareilus et al., 1995). Previous studies employing electrophysiological or imaging techniques (Gesteland et al., 1965; Sicard and Holley, 1984; Firestein et al., 1993, Restrepo et al., 1993) revealed that single ORNs display distinct response profiles; i.e. assaying a range of structurally diverse odorants usually revealed that individual cells either did not respond to any of them or responded only to distinct odorants. To address the question of how individual ORNs are able to detect a structural difference of only one hydrogenated carbon atom, we set out to investigate the responsiveness of isolated ORNs to aliphatic aldehydes with a chain length varying between five and 10 carbon atoms, in particular, to compounds that share the same functional group and only differ in the length of their carbon chain. This allows systematic monitoring of the responsiveness of an individual ORN to slightly different odorous molecules and the assessment of whether ORNs are responsive to stimulation with aliphatic aldehydes of varying chain length (5–10 hydrogenated carbon atoms) was investigated by means of Ca\(^{2+}\) imaging. More than half the cells examined were responsive to aliphatic aldehydes. Individual cells did not react or reacted to one or multiple aldehydes; in the latter case, cells only reacted to aldehydes of consecutive carbon chain lengths. The largest proportion of cells responded to octanal. It was also demonstrated that a structural difference as small as one hydrogenated carbon atom was detectable by the olfactory neurons.

**Materials and methods**

**Odorants**

- n-Pentyl aldehyde (pentanal), n-hexyl aldehyde (hexanal), n-heptyl aldehyde (heptanal), n-octyl aldehyde (octanal), n-nonyl aldehyde (nonanal) and n-decyl aldehyde (decanal) were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

For Ca\(^{2+}\) imaging, odorant solutions were prepared as 100 mmol l\(^{-1}\) stock solutions in dimethyl sulphoxide (DMSO), and these were diluted in methanol on the day of the experiment to a concentration of 1 and 10 mmol l\(^{-1}\). The final concentration was achieved by dilution with Dulbecco’s phosphate-buffered saline (DPBS) immediately before stimulation. At 30 μmol l\(^{-1}\) odorant, the DMSO concentration in solution was 0.003 % (v/v) and the methanol concentration was 0.1 % (v/v). Controls were performed using the highest DMSO and methanol concentrations; at these concentrations, DMSO and methanol did not affect the reactions.

For the determination of cyclic AMP and inositol trisphosphate (InsP\(_3\)) concentrations, the 100 mmol l\(^{-1}\) stock solutions were diluted with stimulant buffer to a final concentration of 25 μmol l\(^{-1}\). The solutions were thoroughly mixed in an ultrasonic waterbath at 37 °C and used immediately.
The acetoxyethyl esters of Fluo-3, Fura Red and the detergent Pluronic F-127 were obtained from Molecular Probes Europe (Leiden, The Netherlands). DPBS, collagenase type II, cysteine, concanaclavin A and bovine serum albumin were purchased from Sigma Chemical Co. (Deisenhofen, Germany). The cyclic AMP and InsP3 radioligand assay kits were purchased from Amersham.

**Animals**

Rats *(Rattus norvegicus)* were supplied by Charles River or were bred in the Institute.

**Isolation of single rat sensory neurons**

The whole olfactory epithelium was dissociated as described by Tareilus et al. (1995). After incubation for 5 min at 30 °C in DPBS (pH 7.4) containing 1 mmol l$^{-1}$ Ca$^{2+}$ and collagenase type II, the tissue was gently triturated. The resulting suspension was filtered through nylon mesh with a pore size of 90 μm, and the cells were collected by centrifugation. The pellet was resuspended in ice-cold DPBS, and the cell suspension was placed onto a glass coverslip coated with concanavalin A. To check viability, cells that did not react to any tested odorant were depolarised with DPBS containing 25 mmol l$^{-1}$ KCl. Living olfactory neurons should display a Ca$^{2+}$ signal induced by depolarisation (Maue and Dionne, 1987; Trombley and Westbrook, 1991).

**Ca$^{2+}$ imaging**

Isolated olfactory cells were loaded with a combination of the acetoxyethyl esters of the fluorescent Ca$^{2+}$ indicators Fluo-3 and Fura Red (Tareilus et al., 1995). Intracellular Ca$^{2+}$ levels were measured with a confocal laser scanning microscope (MRC 600, Bio-Rad; Labophot, Nikon). An IBM-compatible personal computer was used to control the imaging system, data acquisition and storage. An argon ion laser with two prominent excitation wavelengths was used as a light source. The intensity of the laser light was controlled by means of different neutral density filters. The fluorescent dyes were excited by the 488 nm line of the argon laser, and the fluorescent light emitted by the cells was imaged under 40× magnification. The Fluo-3 fluorescence (recorded at 525 nm) increases at high Ca$^{2+}$ concentration and decreases at low Ca$^{2+}$ concentration, whereas the Fura Red fluorescence (recorded at 600 nm) displays an opposite response. The fluorescent light emitted by the Ca$^{2+}$ indicators was separated using two filter combinations (filter 1, 495 FG 03-5Q; filter 2, 585 FD 62-5Q, 495 FG 03-5Q) and detected by two photomultipliers. The ratio of the emission intensities of the two fluorescence dyes was then calculated. The measurement software performs the ratio calculation of the fluorescence intensities from the two indicators as closed curves and produces a pseudocolour image for the intensity of each fluorescent dye. During the experiment, a perfusion system was used to exchange the extracellular medium (Lipp and Niggli, 1993; Tareilus et al., 1995).

**Isolation of olfactory cilia**

Preparations of chemosensory cilia from rat olfactory epithelia were obtained as described by Anholt et al. (1986) and Chen et al. (1986). All operations were performed at 0–4 °C. Rat olfactory cilia were dissected and washed in Ringer’s solution (120 mmol l$^{-1}$ NaCl, 5 mmol l$^{-1}$ KCl, 1.6 mmol l$^{-1}$ K$_2$HPO$_4$, 1.2 mmol l$^{-1}$ MgSO$_4$, 25 mmol l$^{-1}$ NaHCO$_3$, 7.5 mmol l$^{-1}$ glucose, pH 7.4). The cilia were detached using a Ca$^{2+}$ shock procedure in which the medium was changed to Ringer’s solution containing 10 mmol l$^{-1}$ CaCl$_2$. After stirring for 20 min, the deciliated epithelia were removed by centrifugation at 7700 g for 10 min. The supernatant was collected, and the pellet was resuspended and incubated for 20 min in a solution containing 10 mmol l$^{-1}$ CaCl$_2$. The deciliated epithelia were precipitated by centrifugation, and the supernatants containing the detached cilia were combined and centrifuged at 27 000 g for 15 min. The pelleted cilia were suspended in 10 mmol l$^{-1}$ Tris-HCl, 3 mmol l$^{-1}$ MgCl$_2$, 2 mmol l$^{-1}$ EGTA, pH 8.0, and stored at −70 °C.

**Stimulation of cilia samples and determination of cyclic AMP and InsP3 concentrations**

Cilia were incubated with aldehydes (25 μmol l$^{-1}$) at 37 °C. After a 2 min incubation, the reaction was stopped by adding ice-cold perchloric acid (7 %). The samples were neutralized by adding a 1:1 (v/v) mixture of 1,1,2-trichlorofluorethane and

![Fig. 1. Chemical structure of the aliphatic aldehydes. The number of carbon atoms in the chain (C5–C10) is used as an abbreviation in the following figures.](image)

The cyclic AMP and InsP3 radioligand assay kits were purchased from Amersham. The cyclic AMP and InsP3 radioligand assay kits were purchased from Amersham.
tri-\textit{n}-octylamine, followed by thorough mixing. After centrifugation (10 min, 1500 g), three phases were obtained; the upper phase, which contained all water-soluble components, was used for second messenger determinations. Cyclic AMP concentrations were determined following the procedure of Steiner et al. (1972). InsP₃ concentration was estimated according to the receptor binding assay of Palmer et al. (1989). The protein concentration of the cilia preparations was measured using the method of Bradford (1976).

**Results**

\textit{Percentage of cells responding to aldehydes}

To register Ca²⁺ responses of isolated ORNs, a confocal imaging system (MRC 600, Bio-Rad) attached to a conventional microscope (Labophot, Nikon, Japan) was used. Cells were loaded with a combination of the Ca²⁺ indicators Fluo-3 and Fura Red, as described by Tareilus et al. (1995). The two fluorescent dyes were excited by the 488 nm line of an argon laser. The fluorescent light emitted was detected by two photomultipliers, and the ratio between the two fluorescent intensities, reflecting the intracellular Ca²⁺ concentration [Ca²⁺]ᵢ, was determined. Cells were then stimulated with a series of aliphatic aldehydes with a chain length of 5–10 carbons (Fig. 1) at concentrations of 0.1–10 \textmu mol l⁻¹. Of 104 cells analysed, 55 responded to at least one aldehyde (53 %), whereas 49 ORNs (47 %) showed no odour-induced reactions, but displayed a Ca²⁺ signal in response to a KCl-induced depolarization.

Fig. 2 shows examples of the responses of two isolated ORNs to application of the various aldehydes (each aldehyde is indicated by its carbon number, e.g. octanal is C8; see Fig. 1). The ORN in Fig. 2A responded to C8, C9 and C10, while the neuron in Fig. 2B responded to C7, C8, C9 and C10. This observation demonstrates the selective, but broadly tuned, response of ORNs; i.e. cells can recognize multiple odorants but not all. To address the question of whether the subpopulation of ORNs examined may preferentially respond to one of the tested aldehydes, the percentage of cells responding to each aldehyde at one concentration (5 \textmu mol l⁻¹) was determined. Fig. 3 shows that the proportion of responsive cells was highest for C8 (36 %), indicating a preference for C8, and this was followed by C7 (31 %).

\textit{Do ORNs respond only to aldehydes of consecutive carbon chain lengths?}

The reaction profile in Fig. 2 indicates that both ORNs responded selectively to aldehydes of consecutive carbon chain

![Fig. 2. Response profiles of representative olfactory neurons stimulated with aliphatic aldehydes. Details of aldehydes are given in Fig. 1. The y-axis shows the ratio of fluorescent light intensities emitted by the Ca²⁺ indicators Fluo-3 and Fura Red, reflecting [Ca²⁺]ᵢ (for details, see Materials and methods). (A) This sensory neuron responded to stimulation with C8, C9 and C10, but no responses were recorded to stimulation with C5, C6 or C7. (B) The response profile of a neuron reacting to C7, C8, C9 and C10.}
length (Fig. 2A; C8, C9, C10; Fig. 2B; C7, C8, C9 and C10). To approach the question of whether this is a common feature of ORNs, it was determined whether cells responding to multiple aldehydes would only recognize aldehydes of consecutive carbon chain lengths. It was found that 16 of 16 cells responding to at least two different aldehydes only recognized successive aldehydes. Thirteen of these reacted to at least three different consecutive aldehydes, showing no ‘gap’ in the response profile, e.g. two cells responding to C10 and C5 also recognized all the intermediate aldehydes, C6, C7, C8 and C9, suggesting that ORNs detect aldehydes with successive carbon chain lengths.

**Discrimination of aliphatic aldehydes**

The response profiles shown in Fig. 2 also reveal that the cells did not show a stereotyped response to application of distinct aldehydes; instead, the response amplitude became larger as the chain length of the aldehyde increased, suggesting a higher affinity for C8–C10 aldehydes. In addition, it is noteworthy that the cell shown in Fig. 2A demonstrated no significant response to C7 but a strong response to C8. This observation suggests that individual ORNs may be able to discriminate structural differences as small as a single hydrogenated carbon atom. The percentage of cells that will respond to only one of two successively applied aldehydes (at the same concentration) with varying structural differences was therefore determined (Fig. 4); e.g. 18 of 56 cells responded only to one of two aldehydes with a given difference of one CH₂ group and therefore discriminated the applied odorants. It was found that, with increasing structural difference from one to three CH₂ groups, the proportion of discriminating cells becomes larger. Aldehydes with a structural discrepancy of just one hydrogenated carbon could be discriminated by 32% of cells, whereas 51% of cells could discriminate between aldehydes with a difference of two CH₂ groups and 68% of...
cells could discriminate between two aldehydes when the difference in chain length was three CH$_2$ groups. An increase in chain length above this by four and five CH$_2$ groups was not, however, discriminated by a larger proportion of cells (68%).

To探索 whether there is a correlation between carbon chain length and discrimination, the percentage of discriminating cells for all possible combinations of two sequentially applied aldehydes was determined. The results in Fig. 5 show that the percentage of discriminating cells is higher for C5, C6 and C7: 50% discriminated between C5 and C6 and between C6 and C7, while 27% of cells discriminated between C7 and C8, 15% differentiated between C8 and C9, and 18% discriminated between C9 and C10 (Fig. 5A). The finding of a smaller proportion of cells differentiating between longer-chained aldehydes is supported by results for the discrimination of aldehydes with a structural difference of two hydrogenated carbons (Fig. 5B). The percentage of discriminating cells is smaller for longer carbon chains (discrimination between C5 and C7, 63%; between C6 and C8, 58%; between C7 and C9, 44%; between C8 and C10, 45%). For aldehyde pairs with a structural difference of more than three hydrogenated carbon atoms, the proportion of discriminating cells was between 64% and 80% (data not shown).

**Responses of ORNs at various aldehyde concentrations**

On the basis of the observation that individual cells exhibit different response amplitudes to stimulation with aldehydes of varying carbon chain length (Fig. 2), the influence of the odorant concentration was investigated. Fig. 6 shows the response profiles of two representative ORNs stimulated with increasing odorant concentrations. The neuron in Fig. 6A did not react to a mixture of C8, C9 and C10 and was therefore tuned to one or more members of the mixture C5, C6 and C7 (data not shown), while the cell in Fig. 6B did not respond to C5, C6 and C7, but did react to application of a mixture containing C8, C9 and C10 (data not shown). Comparing the reactions to different concentrations, the ORN shown in Fig. 6A demonstrated no significant response at low concentrations of 0.1 and 1 $\mu$mol l$^{-1}$; however, concentrations of 5 $\mu$mol l$^{-1}$ and above elicited reactions in a dose-dependent manner. Within the range tested, the response amplitude did not saturate. In contrast, the response profile of the ORN in Fig. 6B is quite different: (i) the cell responded to a very low stimulus concentration of 0.1 $\mu$mol l$^{-1}$ and (ii) the response amplitude increased only in the range 0.1–5 $\mu$mol l$^{-1}$, while higher doses did not elicit larger responses. These observations clearly demonstrate that individual ORNs have different concentration thresholds for aldehydes and show dose-dependent reactions within distinct ranges of odorant concentrations.

By determining the percentage of cells reactive to application of aldehydes at various concentrations, it was found that higher odorant doses activated more cells: three out of 19 cells tested (16%) responded at a concentration of 0.1 $\mu$mol l$^{-1}$, 11 of 31 cells (35%) reacted at 1 $\mu$mol l$^{-1}$, and 35 of 54 ORNs (65%) showed a response to 5 $\mu$mol l$^{-1}$ aldehyde.

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**Fig. 6.** Responses of two isolated olfactory neurons stimulated with increasing concentrations of aldehyde mixtures. For further details, see Fig. 2. Concentrations of aldehydes are given (in $\mu$mol l$^{-1}$) below the black bars. (A) This neuron showed significant responses at concentrations of 5 $\mu$mol l$^{-1}$ and above (stimulus mixture C5, C6 and C7). These responses became larger with increasing aldehyde concentration, the cell reacting in a dose-dependent manner. Note that the response amplitude was not saturated at the concentrations used. (B) This sensory cell responded to the lowest concentration of the mixture C8, C9 and C10 tested (0.1 $\mu$mol l$^{-1}$); the response amplitude increased with increasing aldehyde concentration from 0.1 to 5 $\mu$mol l$^{-1}$. Higher concentrations (10 or 20 $\mu$mol l$^{-1}$) did not elicit larger responses.
odorants, each ORN displaying a distinct response profile. It was shown that individual ORNs are able to react selectively, but rather non-specifically, to sets of different odorants (Gesteland et al., 1965; Sicard and Holley, 1984; Getchell, 1987). This view raised questions about the ability of individual ORNs to discriminate structurally related odorous compounds.

The results obtained in the present study, in which olfactory cells were stimulated with a series of aliphatic compounds, indicate that ORNs can discriminate between odorants with minimal differences between their odo-topes. It was found that individual ORNs are capable of responding to multiple odorants that differ only in the length of their carbon chain, thus confirming previous studies using other aliphatic compounds (Sato et al., 1994; Malnic et al., 1999). At a specific odor concentration, ORNs recognize only a distinct range of aldehydes (Fig. 2), indicating that carbon chain length is one of the determinants of the interaction between odorants and receptors. Interestingly, the range of reactive aldehydes consisted only of aldehydes with successive carbon chain lengths (Fig. 2); this has also been observed for aliphatic alcohols and acids (Sato et al., 1994; Malnic et al., 1999). If one assumes that individual ORNs express only one odorant receptor type, as suggested by Malnic et al. (1999), the responsiveness of one cell reflects the receptive properties of one odorant receptor type. Thus, a distinct odorant receptor subtype is activated only by aldehydes having the appropriate chain length; aldehydes with a shorter or longer chain length are unable to activate the receptor. The high proportion of olfactory neurons (53%) responding to the aliphatic aldehydes suggests that simple odorous molecules are able to activate multiple odorant receptor subtypes with different receptive sites. This may be partly because aliphatic molecules are flexible in structure and are thus able to assume different conformations (Amoore, 1970), which means that they are capable of activating more receptor types than less flexible odorous molecules. The flexibility of aliphatic molecules may also provide a possible explanation for the reduced ability of ORNs to discriminate aldehydes with longer aliphatic chains (Fig. 5); these molecules may form more conformations than odorants of shorter carbon chain length.

Concerning the discriminability of odorant pairs with increasing chain length, the results of the present study (Fig. 4) show that, within the range from one to three carbon atoms, the proportion of discriminating cells increases with increasing structural difference. This result is in line with recent psychophysical studies demonstrating a negative correlation between discrimination performance and structural similarity (Laska and Teubner, 1999). Together with the defined range of aldehydes recognized by individual ORNs, the ability to discriminate between odorant pairs emphasizes the importance of carbon chain length as a molecular determinant.

![Graph](image_url)

Fig. 7. Aldehyde-induced changes in cyclic AMP concentrations in rat olfactory cilia. The applied aldehydes elicited different cyclic AMP responses; the responses became larger with increasing chain length from C5 to C8, with C8 being the most effective. Aldehydes with longer carbon chains (C8, C9 and C10) induced greater increases in the cyclic AMP concentration than shorter aldehydes. Values are means ± S.E.M. of three experiments.

**Application of 10 μmol l⁻¹ aldehyde elicited a reaction in 20 of 36 cells tested (56%).**

**Determination of aldehyde-induced changes in second messenger concentrations**

Biochemical approaches revealed that in olfactory cilia preparations different subsets of odorants elicited elevations in the levels of either cyclic AMP or inositol trisphosphate (InsP3) (Boekhoff et al., 1990; Breer et al., 1990; Ronnett et al., 1993). To investigate which transduction cascade is activated by the aliphatic aldehydes and whether all members of the aldehyde set stimulate the same transduction pathway, cilia fractions from rat olfactory epithelium were stimulated by the aldehydes prior to determination of the cyclic AMP and InsP3 concentrations. The concentration of InsP3 was not affected by any of the aldehydes (data not shown), but every aldehyde induced an increase in the cyclic AMP level. As can be seen in Fig. 7, the aldehyde-induced cyclic AMP responses were quite different. From C5 to C8, the increase in chain length correlates with a higher potency for inducing cyclic AMP formation; cyclic AMP signals induced by C9 and C10 were lower. The maximal cyclic AMP response was elicited by C8, which correlates with the observation that the highest percentage of cells respond to this aldehyde (Fig. 3).

**Discussion**

This study provides further insight into the responsiveness of individual olfactory neurons to structurally related odorous compounds, notably to a series of aliphatic aldehydes. Previous studies have characterized the responsiveness of individual ORNs to arrays of structurally very different odorants (Gesteland et al., 1965; Sicard and Holley, 1984; Getchell, 1974). It was shown that individual ORNs are able to react selectively, but rather non-specifically, to sets of different odorants, each ORN displaying a distinct response profile.

These observations have led to the concept that common structural features, such as functional groups, aliphatic chain length, an aromatic ring, polarity, hydrophobicity, etc. (so-called ‘odotopes’) may enable odorous compounds with quite different structural formulae to activate the distinct odorant receptor types expressed in an individual ORN (Shepherd, 1987). This view raised questions about the ability of individual ORNs to discriminate structurally related odorous compounds.
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References


