Mitral and tufted cells differ in the decoding manner of odor maps in the rat olfactory bulb

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Abstract

Mitral and tufted cells in the mammalian olfactory bulb are principal neurons, each type having distinct projection pattern of their dendrites and axons. The morphological difference suggests that mitral and tufted cells are functionally distinct and may process different aspects of olfactory information. To examine this possibility, we recorded odorant-evoked spike responses from mitral and middle tufted cells in the aliphatic acid- and aldehyde-responsive cluster at the dorsomedial part of the rat olfactory bulb. Homologous series of aliphatic acids and aldehydes were used for odorant stimulation. In response to adequate odorants, mitral cells showed spike responses with relatively low firing rates, while middle tufted cells responded with higher firing rates. Examination of the molecular receptive range (MRR) indicated that most mitral cells exhibited a robust inhibitory MRR while a majority of middle tufted cells showed no or only a weak inhibitory MRR. In addition, structurally different odorants that activated neighboring clusters inhibited the spike activity of mitral cells, whereas they caused no or only a weak inhibition in the middle tufted cells. Furthermore, responses of mitral cells to an adequate excitatory odorant were greatly inhibited by mixing the odorant with other odorants that activated neighboring glomeruli. In contrast, odorants that activated neighboring glomeruli did not significantly inhibit the responses of middle tufted cells to the adequate excitatory odorant. These results indicate a clear difference between mitral and middle tufted cells in the manner of decoding the glomerular odor maps.

Keywords: Molecular receptive range, Lateral inhibition, Odorant response
Introduction

The surface of the mammalian olfactory bulb (OB) is covered by a few thousand glomeruli. Individual glomeruli represent a single odorant receptor (OR) (Buck 2000; Mombaerts 1999). Thus the glomerular sheet of the OB forms the maps of ORs (Mori et al. 1999). Within each glomerulus two types of projection neurons, mitral and tufted cells, receive synaptic inputs from the axons of olfactory sensory neurons. Cell bodies of mitral cells are lined in the thin mitral cell layer (MCL) while those of tufted cells are distributed in the external plexiform layer (EPL) and glomerular layer (GL). In addition, the two types of neurons differ in the size of terminal tuft of primary dendrite within the glomeruli, projection pattern of their secondary dendrites, laminar distribution of axon-collaterals within the OB, and the pattern of axonal projection to the olfactory cortex (Figure 1) (Haberly and Price 1977; Mori et al. 1983; Orona et al. 1984; Pinching and Powell 1971; Schoenfeld and Macrides 1984; Schoenfeld et al. 1985; Scott et al. 1980; Sleen and Hall 1977).

Mitral cells and internal tufted cells (or displaced mitral cells) project long secondary dendrites in the deeper half of the EPL (Mori 1987; Mori et al. 1983; Orona et al. 1984). These cells project axons to all regions of the olfactory cortex. Tufted cells in the superficial two-third of the EPL (middle tufted cells) have relatively short secondary dendrites that extend in the superficial half of the EPL. Tufted cells in the GL (external tufted cells) have very short or no secondary dendrites. Middle tufted cells project their axons selectively to the rostromedial regions of the olfactory cortex, which include the anterior olfactory nucleus, ventrorostral part of the anterior piriform cortex, and the olfactory tubercle (Ekstrand et al. 2001; Haberly and Price 1977; Schoenfeld and Macrides 1984; Scott 1981; Sleen and Hall 1977).
The morphological difference between mitral and middle tufted cells suggests that they are functionally distinct and may process different aspects of olfactory information (Macrides et al. 1985; Mori 1987; Shepherd and Greer 1998). The two types of cells might differ in the way of processing the input signals that are encoded in the glomerular sheet. In fact, in vivo physiological analysis showed that tufted cells have lower threshold for spike responses to electrical stimulation of the olfactory nerve (Schneider and Scott 1983). In the slice preparation of the OB, tufted cells show a smaller extent of lateral inhibition following electrical stimulation of the glomeruli (Christie et al. 2001). However, systematic comparison of odorant-evoked responses between the two types of projection neurons has not been reported yet.

For the comparison of the response pattern to given odorants it is ideal to record from mitral and tufted cells innervating a same glomerulus in the OR map and thus receiving a same OR input. Due to the technical difficulties, however, we employed here an indirect method. Because glomeruli with similar molecular receptive range (MRR) are clustered in local regions (Inaki et al. 2002; Takahashi et al. unpublished data; Uchida et al. 2000), we compared the odorant-response patterns among mitral and tufted cells in a same cluster.

Using optical imaging of intrinsic signals, we previously reported a cluster of glomeruli that responded to homologous series of aliphatic acids and aliphatic aldehydes in the dorsomedial part of the rat OB (Uchida et al. 2000). In the present study, we recorded single-unit activity from the MCL and EPL in the dorsomedial cluster, and compared the patterns of odorant-evoked responses between MCL units (mitral cells) and EPL units (a majority of them being middle tufted cells). The results indicate that the two types of projection neurons show a clear difference in the firing rate of odorant-evoked spike responses and in the range of odorants that cause
excitatory and inhibitory responses.
Materials and methods

Thirty two adult Wistar rats (8-12 weeks old; 180-250 g; Japan SLC, Shizuoka, Japan) were anesthetized with urethane (1.2 g/kg) and then prepared for acute electrophysiological recording from the OB according to the method described previously (Kashiwadani et al. 1999). Three rats were anesthetized with medetomidine (0.5 mg/kg), ketamine (67.5 mg/kg) and pentothal sodium (25 mg/kg) and prepared for optical recording of intrinsic signals according to the method described previously (Inaki et al. 2002; Uchida et al. 2000). Animals were mounted in a stereotaxic apparatus (SR-5N, Narishige, Tokyo, Japan). Body temperature was maintained at 37.5 °C by a homeothermic heat pad system (ATB-1100, Nihon Kohden, Tokyo, Japan). All experiments were performed in accordance with the guidelines of the Physiological Society of Japan and the animal experiment committee of Univ. of Tokyo.

For optical imaging of intrinsic signals, the dorsal surface of the OB was exposed and covered with 1.5 % agarose gel and glass slip. The intrinsic signals induced by odorant stimulation were imaged under 705 nm wavelength light illumination (Meister and Bonhoeffer 2001). Images of reflected light from the surface of the OB were collected using CCD camera (SD8310, TELI, Tokyo, Japan) with a tandem-lens macroscope arrangement, digitized and stored with a PC using a frame grabber board (Pulsar, Matrox, Quebec, Canada). The images had a spatial resolution of 320 x 240 pixels (after 2 x 2 binning). In the recording session, the focusing depth of the CCD camera was adjusted to 50-150 µm below the surface of the OB. For each recording trial, data were collected for 8 sec with a frame duration of 500 ms (16 frames/trial). Odorant stimulation was applied from the beginning of the 4th to the end of the 16th
Images were analyzed using IDL software (Research Systems, Boulder, Colorado). Images of odorant-induced responses were obtained by dividing the magnitude of signals acquired during odorant stimulation (frame 10-16) by that acquired before stimulation.

For electrophysiological experiments, dorsal surface of the OB was surgically exposed. For recording single-unit activity, a glass micropipette (10-15 MΩ DC resistance; filled with 4M NaCl) was inserted vertically into the dorsomedial part of the OB. A stainless concentric electrode (diameter: 300 µm) was inserted stereotaxically into the lateral olfactory tract (LOT) for electrical stimulation (2.7 mm anterior from the bregma, 3.2 mm lateral from the midline, and about 6 mm deep form the dorsal surface of the brain.). The electrode tip was located in the LOT at the point about 1.5 mm caudal to the border between the anterior olfactory nucleus and the anterior piriform cortex. The stimulus intensity was set to the minimum voltage that evoked the maximal LOT-evoked field potential (square pulse: 3 – 8 V, 100 µsec duration). The configuration of the LOT-evoked field potential in the OB was used for monitoring the tip position of the recording micropipette (Rall and Shepherd 1968). The recorded action potentials were filtered using a band pass filter (150 Hz - 10 kHz; EW-610J, Nihon Kohden, Tokyo, Japan). Respiratory rhythms were detected using a strain gage attached around the animal’s chest (652-T, Nihon Kohden, Tokyo, Japan). The recorded signals were stored in the computer via AD converter with spike2 software (Cambridge Electronic Design, Cambridge, UK).

For the stimulation of olfactory epithelium, we used homologous series of fatty acids {propionic acid (3COOH), butyric acid (4COOH), valeric acid (5COOH), caproic acid (6COOH), heptylic acid (7COOH), caprylic acid (8COOH), pelargonic acid (9COOH)}, and aliphatic aldehydes {propylaldehyde (3CHO), butylaldehyde
(4CHO), valeraldehyde (5CHO), hexylaldehyde (6CHO), heptylaldehyde (7CHO), octylaldehyde (8CHO), nonylaldehyde (9CHO)). In addition, we prepared a mixture of fatty acids (3-9COOH), a mixture of aldehydes (3-9CHO), a mixture of alcohols (propyl alcohol (3OH), butyl alcohol (4OH), pentyl alcohol (5OH), hexyl alcohol (6OH), heptyl alcohol (7OH), octyl alcohol (8OH), nonyl alcohol (9OH)), a mixture of ketones (propyl methyl ketone (K3-1), butyl methyl ketone (K4-1), pentyl methyl ketone (K5-1), hexyl methyl ketone (K6-1), heptyl methyl ketone (K7-1), di-propyl ketone (K3-3), heptyl propyl ketone (K7-3)), a mixture of phenol and its derivatives (phenol, o-cresol, m-cresol, p-cresol), a mixture of guaiacol and its derivatives (guaiacol, creosol, salicyl aldehyde), and a mixture of cyclic ketones {(+) -carvone, (+)-camphor}. Each odorant was diluted to 10^{-1} (vol/vol) in odorless mineral oil and stored in a glass test tube sealed with a screw cap. Odorant stimulation was performed by placing the opening of the test tube at a distance of 1 cm from the animal’s nostril. To check the concentration, five representative stimulus odorants were sampled at the tip of the animal’s nostril that was 1 cm distant from the opening of the odorant-containing test tube. Gas chromatographic analysis indicated the following concentration of the stimulus odorants: valeraldehyde (5CHO), 12.7 ppm; hexylaldehyde (6CHO), 8.3 ppm; heptylaldehyde (7CHO), 5.0 ppm; valeric acid (5COOH), 1.3 ppm; heptylic acids (7COOH), 0.2 ppm.

For simultaneous application of an adequate odorant and other odorants, the openings of two test tubes (one tube containing an adequate odorant and being placed at the left, and the other tube containing other odorants and at the right) were placed in front of the animal’s nostril (each at a distance of 1 cm). For the control experiment, we placed at the left the test tube containing an adequate odorant and at the right the tube containing odorless mineral oil.
Firing frequency of a recorded cell was calculated with every 100 msec bin width using spike2 software. Spontaneous spikes were recorded for 5 sec before the odorant stimulation. Averaged firing rate of the spontaneous discharges was then calculated. Maximum firing rates (bin width = 100 msec) during adequate odorant stimulation were measured two to five times and averaged. The magnitude of the odorant-evoked response was calculated by subtracting the number of spikes that occurred (during 2 sec) before the stimulus onset from the number of spikes that occurred (during 2 sec) after the stimulus onset. For each odorant, we recorded the odorant-evoked responses two to five times, and calculated the averaged magnitude of the odorant-evoked response ($\Delta$ spikes/sec in figure 4, 5, 7 and 8).

For the estimation of the fluctuation of spontaneous discharges, we counted the number of spikes that occurred from 5 sec to 3 sec and from 3 sec to 1 sec before stimulus onset. The magnitude of the spike frequency fluctuation was then calculated by subtracting the former from the latter. The measurement of the fluctuation was repeated for 15–40 times for each unit. We then averaged the spontaneous fluctuations and determined the standard deviation (SD) of the spontaneous fluctuation for each cell. When odorant responses were greater than 2 times SD (2SD), we considered them significant.

Some neurons were juxtacellularly dye-labeled after single-unit recording using 0.5% Neurobiotin (Vector Laboratories) in pipette solution (Pinault 1996). Positive current was applied for labeling the neurons (5-20 nA consisted of 50 % duty cycle of 200 msec pulses for 10min). More than 30 min after the end of the current application, animals were deeply anesthetized and perfused with 0.9 % NaCl solution followed by 4 % paraformaldehyde (PFA) in phosphate buffer (PB, pH 7.4). Brains were postfixed in 4 % PFA in PB at 4 °C for overnight, coated by egg yolk and kept
in 4 % PFA at 4 °C overnight. Brains were kept in PBS containing 30 % sucrose for 3-5 days for cryoprotection. Coronal sections (50 µm thick) were cut on a freezing microtome (Yamato Ko-ki, Saitama, Japan), thoroughly washed in PBS, and incubated with avidin/biotin peroxidase complex (ABC; Vector Laboratories) for at least 3 h at room temperature. Neurobiotin was revealed by stable DAB (Kirkegaard & Perry Laboratories), using nickel intensification. The images of neurons were captured using Olympus CCD camera with Zeiss microscope (Olympus DP50 and Zeiss Axioplan2).
Results

Unit identification and location

Based on our previous and present data obtained by the optical imaging of intrinsic signals (Figure 6A and B) (Inaki et al. 2002; Takahashi et al. unpublished data; Uchida et al. 2000), we estimated the position of fatty acid- and aliphatic aldehyde-responsive cluster of glomeruli in the dorsal surface of the OB. We then penetrated the recording microelectrode in the cluster and recorded single-unit activity in the GL, EPL and MCL. Since the pattern of LOT-evoked field potential correlates with the histological layers (Rall and Shepherd 1968), we routinely monitored the field potential during the search of single-units. Since the component of the LOT-evoked field potential during period III (Rall and Shepherd 1968) flipover at the MCL, we noted the depth of the flipover point in each penetration. The depth of the recorded single-unit was measured and the distance from the flipover point was calculated.

Based on the recorded position and the responses to LOT stimulation, we classified the recorded single-units into three groups; MCL units, EPL units and GL units. MCL units were located within ± 30 µm (+, superficial; -, deep) from the flipover point. About 60% of the MCL units (21 out of 34 units, shown by asterisks (*) in Figure 3E) showed the short-latency antidromic spike responses to the LOT volley (Mori and Takagi 1978), indicating that they are mitral cells. Two of them were morphologically identified as mitral cells (Figure 2A).

EPL units were located between 50 µm and 250 µm superficial to the flipover point (Figure 3E). A small fraction of the EPL units (6 out of 38 units) showed LOT-evoked antidromic spike responses suggesting that they were recorded from
either mitral cell dendrite or internal tufted cells. These units (shown by asterisks) were often located in the deep part of the EPL (between 50 µm and 120 µm superficial to the flipover point). Therefore, we excluded these units in the following comparative analysis of MCL and EPL units. Since a majority of cell bodies in the EPL are middle tufted cells (Pinching and Powell 1971), we speculate that the most of the EPL units that did not show antidromic spike response originate from middle tufted cells. Three of the EPL units were morphologically identified as middle tufted cells (Figure 2B).

GL units were located more than 250 µm superficial to the flipover point. Since cells in the GL are heterogeneous (Kosaka et al. 1998), we did not analyze these units in the present study.

**Firing rate**

To compare the odorant-response properties between mitral and middle tufted cells, we examined odorant-evoked spike responses of EPL units and MCL units. Figures 3A and B show odorant-evoked response pattern of two representative EPL units. These EPL units showed high frequency burst spike responses that were in phase with the respiratory rhythm. Some neurons showed a gradual decrease of spike amplitude during each burst response. MCL units (Figures 3C and D) also showed odorant-evoked burst spike responses, but the firing rate was lower than that of EPL units.

In Figure 3E, the averaged maximum firing rate of odorant-evoked spike discharges (filled circles) and the averaged firing rate of spontaneous discharges (open squares) of the recorded units were plotted against the laminar position of single-units (that was represented as the distance from the flipover point). The maximum firing rates
of MCL units ranged from 33 to 100 Hz (60.8 ± 14.5; Mean ± SD, n=34), while those
of EPL units ranged from 50 to 376.7 Hz (161.1 ± 78.3; Mean ± SD, n=32). Averaged firing rates during burst discharges of MCL and EPL units in response to
the odorant stimulation were 41.5 ± 13.1 and 103.0 ± 63.8 (Mean ± SD), respectively.
Both of the averaged maximum firing rates and the averaged firing rates of EPL units
were significantly higher than those of MCL units (P < 0.0001; standard Student
t-test). Spontaneous firing rates of MCL units and EPL units were 7.4 ± 6.6 and 16.9
± 21.1 (Mean ± SD), respectively. These results indicate that a majority of EPL
units respond to odorants with high firing rates (maximum firing rates were more than
100 Hz). In contrast, MCL units respond to the same stimulus odorants with
relatively low firing rates (maximum firing rates were less than 100 Hz).

Molecular receptive range (MRR) properties of EPL and MCL units

Individual mitral/tufted cells in the dorsomedial part of the rabbit OB respond with
increased spike discharges to a range of fatty acids and aldehydes with similar carbon
numbers (Imamura et al. 1992; Mori et al. 1992). In addition, mitral/tufted cells
receive inhibitory inputs by a defined subset of odorants with structures closely
related to the excitatory odorants (Yokoi et al. 1995). In the present study, we
compared the MRR properties (Mori and Yoshihara 1995) between EPL units and
MCL units (Figure 4). Homologous series of fatty acids (3-9COOH) and aliphatic
aldehydes (3-9CHO) were used to examine the excitatory and inhibitory MRR of
neurons in the dorsomedial cluster.

Figure 4A1 shows responses of an EPL unit to a homologous series of aliphatic
aldehydes. Following stimulation with adequate odorants {valeraldehyde (5CHO)
and hexylaldehyde (6CHO)} this EPL unit showed high frequency burst spike
responses that were in phase with the respiratory rhythm. Except for a transient inhibitory response (e.g., brief pause of burst discharges after the initial burst response to valeraldehyde (5CHO) stimulation in A1), we did not observe significant overall inhibition of spike discharges.

For each odorant, we recorded the odorant-evoked responses two to five times, and calculated the averaged magnitude of the odorant-evoked responses. The MRR of this unit for the homologous series of aldehydes is shown by plotting the averaged magnitude of the odorant-evoked responses (Figure 3A2). Excitatory responses were shown by open bars, while inhibitory responses by filled bars.

An example of response pattern of MCL units is shown in Figure 4B1. The MCL unit responded strongly to valeraldehyde (5CHO) and hexylaldehyde (6CHO). In addition, the MCL unit showed inhibitory responses to the butylaldehyde (4CHO) and nonylaldehyde (9CHO). The MRR profile of the MCL unit is summarized in Figure 4B2. The excitatory MRR of the MCL unit covered valeraldehyde (5CHO) and hexylaldehyde (6CHO), while inhibitory MRR included propylaldehyde (3CHO), butylaldehyde (4CHO) and nonylaldehyde (9CHO).

Figure 5 demonstrates representative MRR profiles of EPL units (A-J) and MCL units (K-T). Comparison of the MRR profiles for the aldehydes or acids between the EPL units and the MCL units showed two characteristic differences. Firstly, many EPL units showed no or only a weak inhibitory MRR, whereas most MCL units exhibited robust inhibitory MRR. Twenty out of 26 MCL units (76.9 %) showed significant inhibitory MRR, whereas only 5 out of 24 EPL units (20.8 %) showed significant inhibitory MRR. Four out of the 5 EPL units that showed significant inhibitory MRR were located in the deep region of the EPL (between 50 µm and 120 µm superficial to the flipover point).
Secondary, individual MCL units showed a sharply tuned excitatory MRR, whereas individual EPL units showed a wider excitatory MRR (Figure 5). The excitatory MRR of MCL units covered $2.0 \pm 1.3$ odorants (mean ± SD; n=27) in average, whereas that of EPL units covered $3.2 \pm 1.4$ odorants (mean ± SD; n=24) for the homologous series of fatty acids or aldehydes.

Inhibitory input from neighboring clusters

Optical imaging studies from the dorsal OB (Figure 6) (Inaki et al. 2002; Takahashi et al. unpublished data; Uchida et al. 2000) showed that the fatty acid- and aliphatic aldehyde-responsive cluster (cluster $A$ in Figure 6) was surrounded by neighboring clusters (cluster $B$, $C$ and $D$) with different odorant-tuning specificity. Most glomeruli in cluster $B$ respond to aliphatic alcohols (Figure 6C) and aliphatic ketones. Glomeruli in cluster $C$ are typically activated by phenol and its derivatives (Figure 6D), and by guaiacol and its derivatives (Figure 6E). Cluster $D$ glomeruli respond to aliphatic and cyclic ketones (Takahashi et al. unpublished data).

Mitral cells extend long secondary dendrites tangentially in virtually all directions (Figure 1) (the mean length is about $1.2 \times 10^3 \ \mu m$ in rabbits) (Mori et al. 1983). Thus Mitral cells may receive inhibitory inputs via local interneurons not only from mitral/tufted cells in the same cluster but also from those in the neighboring clusters. In contrast, secondary dendrites of middle tufted cells are relatively short (the mean length is about $0.8 \times 10^3 \ \mu m$ in rabbits) and tend to extend in particular directions. Thus middle tufted cells may receive no or only a small inhibitory input from mitral/tufted cells in the neighboring clusters. To examine these possibilities, we recorded MCL units and EPL units in the cluster $A$ and checked whether they were inhibited by odorants that activated neighboring clusters, $B$, $C$ and $D$. To activate
cluster B glomeruli, we used a mixture of aliphatic alcohols or a mixture of aliphatic ketones (Figure 7A). A mixture of phenols or a mixture of guaiacol, creosol and salicyl aldehyde was used to stimulate glomeruli in cluster C. To activate cluster D glomeruli, we used a mixture of aliphatic ketones or a mixture of cyclic ketones. Optical imaging study showed that each of these mixtures activated respective clusters but rarely activated glomeruli in the cluster A (Figure 6).

Figures 7B and C show examples of the response pattern of MCL units. The MCL unit in B showed a strong excitatory response to nonylaldehyde (9CHO). Spike discharges of this unit were strongly inhibited by the mixture of alcohols and by the mixture of aliphatic ketones. In addition, the spike discharges were inhibited weakly by the mixture of phenols. The MCL unit in C was activated by heptylaldehyde (7CHO) and inhibited strongly by a mixture of guaiacol, creosol and salicyl aldehyde. Among the 16 MCL units examined, 56.3 % of them showed significant inhibitory responses to at least one of the five mixtures.

Figures 7D and E show the response pattern of EPL units. In contrast to MCL units, only a small percentage of EPL units showed significant inhibitory responses to the mixtures of odorants that activated the neighboring clusters. Among the 11 EPL units examined, two of them (18.2 %) showed significant inhibitory response to at least one of the above mixtures. The 2 EPL units, which showed the inhibitory responses, were located in the deep region of the EPL.

Response pattern of EPL units and MCL units to the mixture of excitatory and inhibitory odorants

The clear difference in the extent of the inhibitory MRR between EPL units and MCL units suggests that they differ in their response pattern to the mixture of
odorants that activate their own glomerulus and odorants that activate neighboring glomeruli. To examine this hypothesis, we first determined the odorant that activated the recorded unit most strongly among the homologous series of fatty acids or aliphatic aldehydes. We then compared the spike response to the adequate excitatory odorant with the response to the mixture of the adequate excitatory odorant and the remaining fatty acids (or remaining aliphatic aldehydes).

The MCL unit in Figure 8A was activated strongly by the simultaneous application of heptylic acid (7COOH) and odorless mineral oil (A1). However, the MCL unit showed only a weak response to the simultaneous application of heptylic acid (7COOH) and the mixture of the remaining fatty acids {Fatty acids (-7COOH)} (Figure 8A2 and A3). Three MCL units showed complete suppression of the spike response to the adequate excitatory odorant when the excitatory odorant was mixed with the remaining fatty acids (or aldehydes) (data not shown). Compared to the response to the adequate odorant, 6 out of 7 MCL units showed a significant decrease (P < 0.05) in the magnitude of response to the simultaneous application of adequate odorant and the mixture of remaining odorants.

We also examined the responses of MCL units to the mixture of an adequate excitatory odorant and those odorants that activated glomeruli in the neighboring clusters. Compared to the response to the adequate excitatory odorant, the response of MCL units to the mixture was significantly reduced (n=3, data not shown).

Figure 8B shows an EPL unit that responded strongly to heptylic acid (7COOH). The odorant-evoked burst discharge of the EPL unit was in phase with the inspiration of the odorants. In contrast to the MCL units, the EPL unit did not show clear suppression of spike responses when the nose was stimulated with the simultaneous application of heptylic acid (7COOH) and other fatty acids {Fatty acids (-7COOH)}. 
In only 1 out of 6 EPL units, the simultaneous application of the mixture caused a significant decrease in the magnitude of response to the adequate odorant. Two out of the 6 EPL units showed even an augmentation of the excitatory response when we applied the adequate odorant and other odorants.

These results suggest that activation of neighboring glomeruli causes a significant inhibitory effect on the responses of MCL units to the adequate odorant. In contrast, activation of neighboring glomeruli causes little or only a weak effect on the response of EPL units to the adequate odorant.
Discussion

*Different firing rates of mitral and tufted cells*

Present results showed a clear difference between EPL units and MCL units in the maximum firing rates of odorant-evoked responses. Following stimulation with the most effective aliphatic aldehyde or acid, a majority of EPL units in cluster A responded with high firing rates (more than 100 Hz), while MCL units in the same cluster responded with relatively low firing rates (less than 100 Hz). These results suggest that mitral and middle tufted cells may use different firing rates for transmitting the olfactory signals to their target neurons.

A large subset of middle tufted cells, but no mitral cells, express cholecystokinin (CCK), one of the neuroactive peptides of gastrin family (Seroogy et al. 1985). The CCK-expressing middle tufted cells form dendrodendritic synapses in the superficial part of the EPL with the granule cells. Electron microscopic study showed that CCK-positive axon-collaterals of middle tufted cells form asymmetric synapses on the dendrites of GABAergic granule cells in the OB (Liu and Shipley 1994). Since the release of CCK from the synaptic terminals typically require high firing rates (Ghijsen et al. 2001), the high frequency burst discharges might be necessary for middle tufted cells to effectively release CCK to their target neurons.

The maximum firing rates of MCL units were relatively low and ranged from 33 to 100 Hz (mean 60.8 Hz). The averaged firing rate of odorant-evoked burst discharges (41.5 Hz) of MCL units roughly corresponds to that of the γ-range oscillations of local field potentials in the EPL (Adrian 1950; Kashiwadani et al. 1999). Previous studies showed that spike activity of mitral cells is in-phase with the oscillatory local field potential in the EPL and that specific pairs of mitral cells exhibit synchronized spike
discharges during odorant stimulation (Kashiwadani et al. 1999). It has been suggested that the synchronized discharges of mitral cells are necessary for olfactory cortical neurons as coincidence detector to effectively integrate signals from many mitral cells. Controlling the spike discharge rates in the $\gamma$-range frequencies might be important for mitral cells to synchronize their activity.

*Mitral and tufted cells decode different aspects of odorant information*

MCL units and EPL units in cluster A shared the property of being activated by a range of aliphatic odorants with similar carbon number (Figure 4). An individual neuron is presumably activated by olfactory axon input to its own glomerulus (the glomerulus that its primary dendrite innervates). However, MCL units and EPL units differ clearly in the response to the odorants that activate neighboring glomeruli. MCL units were typically inhibited by odorants that activate neighboring glomeruli in cluster A (Figure 5), and by those that activate glomeruli in neighboring clusters (Figure 7). The long secondary dendrites of mitral cells and their extensive dendrodendritic synaptic connections with granule cells are in good agreement with the intensive lateral inhibition. In contrast to mitral cells, the secondary dendrites of middle tufted cells are relatively short (Mori et al. 1983; Orona et al. 1984), and EPL units showed a relatively weak inhibitory response to the odorants that activated neighboring glomeruli.

In the presence of odorants that activate neighboring glomeruli, the spike response of MCL units to the adequate excitatory odorants was abolished or greatly reduced (Figure 8A). The observation suggests that mitral cell output is determined by the interaction between the excitatory input to its own glomerulus and the activity of many neighboring glomeruli. In other words, mitral cell may detect the contrast of
activity between their own glomerulus and neighboring glomeruli. Since individual glomeruli represent a single OR, each mitral cell may detect the contrast of input from its own OR with inputs from those ORs that neighboring glomeruli represent. Therefore, mitral cells may detect the ratios of different odorants, but may not be able to code the absolute concentration of an odorant.

Compared to the mitral cells, EPL units were less influenced by the odorants that activated neighboring glomeruli. Even in the presence of odorants that activated neighboring glomeruli, EPL units strongly responded to the adequate excitatory odorants (Figure 8B). The observation suggests that an individual middle tufted cell can code the presence of odorants that activates its own glomerulus regardless of the presence of odorants that activate neighboring glomeruli. Since middle tufted cells have lower threshold for spike response to olfactory nerve input (Schneider and Scott 1983), they might be able to detect low concentration of odorants. Thus, middle tufted cells might be functionally specialized to detect the presence of specific odorants even at relatively low concentrations among a complex mixture of many odorants that occur in the natural environment.

Despite the presence of intensive dendrodendritic synaptic connections with granule cells, middle tufted cells showed relatively small or no lateral inhibition (Figures 4, 5 and 7). These results suggest that the dendrodendritic synaptic interactions may participate in shaping the middle tufted cell responses in the mode other than the lateral inhibition. It is interesting to note that many middle tufted cells responded with intensive burst discharges during the inhalation phase, whereas the spike-response was temporarily inhibited during the exhalation phase (Figure 3B, 4A1 and 8B1-2). This suggests that the dendrodendritic synapses on middle tufted cells might be responsible for the temporal shaping of spike responses in accord with the
sniffing and respiration rhythms.

In agreement with the previous report (Ezeh et al. 1993), present results suggest that activation of mitral cells does not cause a significant inhibition of middle tufted cells in the same cluster. Mitral cell and middle tufted cells may preferentially interact with distinct subsets (type II and type III) of granule cells.

**Mitral cell and middle tufted cell pathways to the olfactory cortex**

Present results suggest that mitral cells and middle tufted cells handle different aspects of odorant information. The two types of projection neurons show also distinct pattern of axonal projection to the olfactory cortex (Haberly and Price 1977; Schoenfeld and Macrides 1984; Scott et al. 1980; Skeen and Hall 1977). Mitral cells project their axons to the entire part of the olfactory cortex (anterior olfactory nucleus, olfactory tubercle, anterior and posterior piriform cortices, amygdaloid cortex, entorhinal cortex), while tufted cells project axons only to the anterior regions of the olfactory cortex (anterior olfactory nucleus, olfactory tubercle, rostroventral part of the anterior piriform cortex).

The segregation of information into mitral cell and middle tufted cell pathways suggests that different olfactory cortical regions may be functionally distinct and process distinct aspects of olfactory information. The anterior regions of the olfactory cortex may handle information carried by both the mitral cell pathway and middle tufted cell pathway. In contrast, the remaining posterior regions of the olfactory cortex may process information carried only by mitral cell pathway. Therefore, understanding of the functional specificity of mitral cell pathway and middle tufted cell pathway may provide a basis for studying the functional difference among several distinct regions in the olfactory cortex.
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Figure Legends

Figure 1

A schematic diagram illustrating the morphological difference between mitral cells and middle tufted cells. Mitral cells have a relatively large cell body in the mitral cell layer (MCL). They extend long secondary dendrites in the deeper half of the external plexiform layer (EPL) and form dendrodendritic reciprocal synapses with the dendrites of granule cells (type II). They project their axons to the entire part of the olfactory cortex. Middle tufted cells have a relatively small cell body and are scattered in the EPL. They extend relatively short secondary dendrites to the superficial half of the EPL, and form dendrodendritic reciprocal synapses with granule cells (type III). Tufted cells project their axons only to the anteromedial part of the olfactory cortex. ONL, olfactory nerve layer; GL, glomerular layer; IPL, internal plexiform layer; GCL, granule cell layer.

Figure 2

Morphological identification of an MCL unit (A) and an EPL unit (B) using the single-cell staining method (Pinault 1996). A, An MCL unit was labeled juxtacellullarly after examination of its odorant-response property. This cell showed the antidromic spike response to the LOT-volley. The labeled neuron was morphologically identified as a mitral cell. B, A juxtacellullarly labeled EPL unit. This cell was located at 190 µm superficial to the MCL. The labeled cell was morphologically identified as a middle tufted cell.

Figure 3
Comparison of the firing frequency of odorant-evoked spike discharges between EPL units and MCL units.  

A and B, Odorant-evoked spike responses of two EPL units.  C and D, Odorant-evoked responses of two MCL units.  Upper panel shows the spike-frequency histogram (one bin is 100 msec).  Lower trace shows the respiratory rhythm (upward deflection is inhalation).  Bar shows the period of odorant stimulation.  E, Plots of averaged maximum spike rates of odorant-evoked response (filled circles) and spontaneous spike rates (open squares) for MCL-, EPL-, and GL-units.  X axis indicates the distance between the position of single-units and the flipover point (0 µm).  Many EPL units showed high frequency spike responses (more than 100 Hz; broken lines) during odorant stimulation, while MCL units responded with relatively low firing rate (less than 100 Hz; solid lines).

Figure 4

Molecular receptive range (MRR) property of an EPL unit and an MCL unit.  The response pattern of an EPL unit and an MCL unit to a homologous series of aliphatic aldehydes are shown in A1 and B1, respectively.  The excitatory and inhibitory MRRs of these units are shown in A2 and B2.  Each column indicates the magnitude of the change in spike rates (Δ spikes/sec; open bar, an increase; filled bar, a decrease) during odorant-stimulation compared with the spike rate during pre-stimulation period.  Stimulus odorants are propylaldehyde (3CHO), butylaldehyde (4CHO), valeraldehyde (5CHO), hexylaldehyde (6CHO), heptylaldehyde (7CHO), octylaldehyde (8CHO) and nonylaldehyde (9CHO).  Dotted lines indicate the 2SD of fluctuation of spontaneous spike discharges.  Significant excitatory responses and inhibitory responses (greater than the 2SD level) are shown by (+) and (-), respectively.
Figure 5

Comparison of the MRR properties between EPL units (A-J) and MCL units (K-T). Excitatory MRR is shown by open bars with (+), while inhibitory MRR by filled bars with (-). EPL units (A-J) show relatively wide excitatory MRR (3.2 ± 1.4 odorants; mean ± SD, n=24), while MCL units (K-T) have narrow excitatory MRR (2.0 ± 1.3 odorants; mean ± SD, n=27). A majority of MCL units show robust inhibitory MRR, while only a small percentage of EPL units show clear inhibitory MRR.

Figure 6

Optical images of intrinsic signals recorded from the dorsal surface of the rat OB. Olfactory epithelium was stimulated with a mixture of fatty acids (A), a mixture of aldehydes (B), a mixture of alcohols (C), a mixture of phenol and its derivatives (D), and a mixture of guaiacol and its derivatives (E). Fatty acid- and aliphatic aldehyde-responsive cluster (cluster A) is indicated by broken line. Scale bar = 500 µm

Figure 7

Responses of MCL units and EPL units to the odorants that activate neighboring clusters. A, the dorsolateral view of the left olfactory bulb. The shaded area is enlarged and shown diagrammatically below. Single-units were recorded from fatty acid- and aldehyde-responsive cluster (cluster A). Positions of neighboring clusters (B, C, and D) are shown together with odorants that are effective in activating each cluster. Glomeruli in cluster B responds to aliphatic alcohols and aliphatic ketons. Cluster C responds to phenol and its derivatives, o-cresol, m-cresol, p-cresol, guaiacol,
creosol, salicyl aldehyde. Cluster D responds to aliphatic and cyclic ketones. To activate the cluster B, C, or D glomeruli, we used a mixture of aliphatic alcohols (alcohols in A), a mixture of aliphatic ketones (ketones), a mixture of phenol, o-cresol, m-cresol and p-cresol (phenols), a mixture of guaiacol, creosol and salicyl aldehyde (guaiacols) or a mixture of (+)-carvone and (+)-camphor (cyclic ketones). Open bar indicates excitatory response, while filled bar shows inhibitory response. B and C; MCL units. D and E; EPL units.

Figure 8

Responses of an MCL unit (A) and an EPL unit (B) to the mixture of the adequate excitatory odorant and those odorants that activate neighboring glomeruli. Upper plots show raster display of spike discharges, and lower histograms indicate firing rate. The period of odor stimulation is shown by filled bars. A, MCL unit. A1, Responses to the simultaneous application of heptylic acid (\(7\text{COOH}\)) and odorless mineral oil (OIL) (control). A2, Responses to the simultaneous application of heptylic acid (\(7\text{COOH}\)) and other fatty acids. B, EPL unit. B1, Responses to the simultaneous application of heptylic acid (\(7\text{COOH}\)) and odorless mineral oil. B2, Responses to the simultaneous application of heptylic acid (\(7\text{COOH}\)) and other fatty acids. A3 and B3 show the averaged firing rate of responses to the simultaneous application of heptylic acid (\(7\text{COOH}\)) and odorless mineral oil (left column), and that to the simultaneous application of heptylic acid (\(7\text{COOH}\)) and other fatty acids (right column). Error bar: standard error of the mean. Statistical significance was determined using standard Student’s \(t\)-test. ***: \(P<0.0001\).
References


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Figure 1

- ONL
- GL
- EPL
- MCL
- IPL
- GCL
- Periglomerular cell
- Mitral cell
- Middle tufted cell
- Type II Granule cell
- Type III Granule cell
- To anteromedial part of the olfactory cortex
- To entire part of the olfactory cortex
Figure 2

A. MCL unit
Mitral cell

B. EPL unit
Middle tufted cell

GL
EPL
MCL

100 μm
During odorant stimulation
Spontaneous discharge
- : Firing rate = 100Hz
--- : Firing rate > 100Hz
Figure 4

**EPL unit**

**MCL unit**

A1

B1

A2

B2

Δ spikes / sec

3CHO 4CHO 5CHO 6CHO 7CHO 8CHO 9CHO

3CHO 4CHO 5CHO 6CHO 7CHO 8CHO 9CHO
Figure 6

- Fatty acids
  - Cluster A

- Aldehydes
  - Cluster A

- Alcohols
  - Cluster B

- Phenols
  - Cluster C

- Guaicols
  - Cluster C

Scale: 0.10 %, 0.05 %, 0.015 %, 0.005 %
Figure 8

A MCL unit

B EPL unit

Graph A3

Graph B3