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Responses of the Rat Olfactory Epithelium to Retronasal Air Flow

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Abstract

Responses of the rat olfactory epithelium were assessed with the electroolfactogram while odorants were presented to the external nares with an artificial sniff or to the internal nares by positive pressure. A series of seven odorants that varied from very polar, hydrophilic odorants to very non-polar, hydrophobic odorants were used. While the polar odorants activated the dorsal olfactory epithelium when presented by the external nares (orthonasal presentation), they were not effective when forced through the nasal cavity from the internal nares (retronasal presentation). However, the non-polar odorants were effective in both stimulus modes. These results were independent of stimulus concentration or of humidity of the carrier air. Similar results were obtained with multiunit recording from olfactory bulb. These results help to explain why human investigations often report differences in the sensation or ability to discriminate odorants presented orthonasally vs. retronasally. The results also strongly support the importance of odorant sorption in normal olfactory processes.

Introduction

Olfactory stimuli entering the nasal cavity from the back are referred to in the human psychophysical literature as “retronasal” stimuli. This route of stimulation is distinguished from the “orthonasal” route (entering the nose by inspiration through the external nares). Retronasal olfaction stimulation is an element of flavor along with taste and chemesthesis. The flavor of many materials change if the nose is plugged (Murphy and Cain, 1980; Murphy et al., 1977). Psychophysical investigation show that differences in detection accuracy by the two routes can be overcome by training (Pierce and Halpern, 1996). However, odorant names trained retronasally were better identified orthonasally than vice versa (Sun and Halpern, 2005).

Rozin (1982) considered olfaction a dual modality sensing either external objects or objects in the mouth. He proposed three possible mechanisms for that dual function. (1) Taste or trigeminal information from the mouth might tell the brain to interpret the olfactory sensation as coming from the mouth. (2) The interpretation of an olfactory stimulus as coming from the mouth might simply result from the combination of olfactory, taste, and trigeminal information. (3) The olfactory stimulus itself might be different because of the way in which it enters the olfactory region.

Several authors (Sun and Halpern, 2005; Heilmann and Hummel, 2004; Pierce and Halpern, 1996; Small et al., 2005) have suggested that Rozin's third mechanism acts by differential sorption of odorants to the wall of the oral and nasal cavities as described for the frog olfactory epithelium (Mozell and Jagodowicz, 1973; Mozell et al., 1987; 1991). Diaz (2004) reported a

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strong correlation of the odorant air-water partition coefficient with the relative efficacy of a series of odorants for orthonasal and retronasal olfaction. In an elegant study, Frasnelli et al. (2005) used mass spectrometry to report that intranasal profiles of concentration differ across flavor compounds during intraoral presentation, with a polar odorant (diacetyl) achieving lower concentrations in the olfactory cleft region than less polar odorants (ethyl butyrate and ethyl hexanoate). That study is limited because the sampling procedure required flow rates of 100 ml/min through tubes in the nose. This can disturb the local air flow, and one cannot be sure that the tubes reached into the narrow headspace directly over the receptors in the olfactory cleft. Zhao et al. (2004; 2006) modeled air flow in the human and rat nose and suggested that, although they differ in the amount of turbulent flow, both systems should have differences in odorant access to the olfactory regions between inspiratory and expiratory phases. They further suggested that these differences could differentially affect polar odorants and partly account for differences between orthonasal and retronasal olfaction.

We used electroolfactogram (EOG) recordings in the rat to test the prediction that odorant polarity would be a major factor in orthonasal vs. retronasal olfaction. In this preparation, we could directly compare the population response of the receptor sheet. There were responses to only very non-polar, hydrophobic odorants during retronasal presentation. These results confirm the importance of odorant solubility in normal olfactory function. We also tested a similar set of odorant with recordings from the superficial layers of the olfactory bulb in order to provide a parallel measure to the EOG.

Methods

Surgery and EOG recording setup

All animal procedures were consistent with our protocol approved by the Institutional Animal Care and Use Committee of Emory University. The surgery and orthonasal stimulation were similar to our previous reports (Scott-Johnson et al., 2000; Scott et al., 2006). Briefly, male Sprague-Dawley rats (180-300g) were killed by overdose with sodium pentobarbital (200 mg/kg). A Teflon cannula was inserted via the trachea to rest in the retronasal area just over the soft palate. This cannula was tested with a flame ionization gas chromatograph after several uses to ensure that neither the material of the cannula or substances from the animal that adhere to the cannula adsorb any of the odorants used in this study. The bone overlying the dorsomedial nasal recess and the lateral recess at the base of ectoturbinate II was thinned until it could be removed with fine forceps. Recording pipettes filled with Ringer's solution were inserted through the olfactory epithelium until a strong response could be recorded to isoamyl acetate. For the first five experiments, the dorsal electrode arrays were placed seven mm rostral to the fronto-nasal suture and the lateral electrode arrays were placed just above the base of endoturbinate II. For the second five experiments, an additional dorsal electrode was placed more caudally, three mm rostral to the fronto-nasal suture, and an additional lateral electrode was placed just below the base of endoturbinate II. (See figure 1A&B.) Recordings were referenced against a silver wire embedded in EEG electrode paste on the frontal bone. The amplifier frequency band was from 0.1 Hz to 30 Hz. Data were digitized at 1 KHz and were analyzed off line with routines written in MATLAB by one of the authors (JWS).

Odor stimulation

Odorants were introduced with an 8 mm glass manifold connected to bottles containing test stimuli. Odorants were diluted in mineral oil at ratios between 0.1 and 0.001 and kept in 100 ml glass bottles with gas tight Teflon fittings and tubing leading to the stimulus tube. These dilutions were tested with a gas chromatograph equipped with a flame ionization detector when they were made up, and these tests were reconfirmed after several days of use. The mineral oil/odorant mixture covered the bottom of the odor sample bottle for a depth of ~0.5 cm and

had a surface area of about 6 cm^2. Air flowed through the odor sample bottle at a rate of 100 ml/min. The sample was further diluted by injection into an air stream via a glass manifold with multiple ports for the different odorants. There were three presentations of each odorant, and the three responses were averaged. Approximately 60 seconds elapsed between each stimulus presentation. All series contained a blank stimulus to test for contamination or other artifacts. All responses were normalized to the peak response to isoamyl acetate presented orthonasally at a dilution of 0.001 times vapor saturation.

The properties that determine odorant sorption in the nose are not perfectly known. Mozell et al. (1991) used the gas chromatographic retention time on a carbowax column as an estimate, but those numbers are not available for all odorants. We chose odorants to span the range of odorants we have previously used in recordings from the epithelium (Scott and Brierley, 1999; Scott et al., 2000). To estimate hydrophobicity, we used the Hansen solubility parameter (Burke, 1984; Hansen, 1999), which represents the amount of energy required to separate molecules of a compound. This index (calculated with Molecular Modeling Pro v.3.2 software, ChemSW Inc., Fairfield, CA) was useful in predicting the relative response across the epithelium with direct odorant application (Scott, 2006) and the response of odorants with differing nasal air flow rates (Scott et al., 2006). Polarity (the degree of charge separation in the molecule) is one factor in that index. Liquids that are highly soluble in water have high solubility parameters. Therefore we will describe the odorants in this study as being hydrophobic or hydrophilic, although we are aware that other properties may be important in determining the response. The odorants, illustrated in figure 1C, were picked to have a range of hydrophobicity and to have some variation in structure.

For orthonasal stimulation, odorized air flowed in front of the rat's nose at a rate of 1000 ml/min for nine seconds to allow the odorant concentration to stabilize in the system before a vacuum was applied via the tracheal cannula to initiate a two-second sniff at 500 ml/min. For retronasal stimulation, odorized air was applied from the tracheal cannula. In this case air flowed constantly through the glass manifold at a rate of 500 ml/min, but a vacuum port pulled that air away so that it did not enter the nose (see Figure 1B). Odorants were added into the manifold for 9 seconds before the vacuum valve was closed to force odorized air into the nose via the cannula. The stimulus period was 2.5 seconds, followed by inspiration at 500 ml/min. Orthonasal stimuli were retested after the retronasal series to be sure that no electrode had moved. The orthonasal odor tube was set back about 1 cm from the rat's nose to avoid forcing odor into the nose before sniff onset. In contrast, the retronasal flow system was tightly closed and depended on forcing air into the trachea. As a consequence of the different flow rates, the effective concentration of the retronasal stimulus was double that of the orthonasal stimulus. This issue is addressed in some of the experiments. The stimulus control valves were controlled by a program written in LABVIEW.

Multiunit recording

Since there are uncertainties about the exact relationship of the EOG to activity in the olfactory receptor neurons and to the activity in olfactory bulb, we also performed recordings from the olfactory bulb in urethane (1.5 gm. /kg.) anesthetized rats. We chose multiunit recording methods to avoid bias from the inputs to a single cell. The 4×4 electrodes arrays from NeuroNexus provided sampling from populations of cells over a spatial extent of nearly 400 μ by 150μ (see figure 2A). This should be greater than 2 or 3 times the size of a single glomerulus. Similar series of stimuli were presented. At least three, sometimes six, presentations, were made of each stimulus orthonasally, then retronasally, and then retested orthonasally.

These recordings were collected from two sites simultaneously in each animal as indicated in figure 2B. One array was placed on the dorsal lateral curvature of the bulb. This site was chosen

because the nerve and glomerular layers are very thin at the most dorsal region. Although we used photographs of the bulb surface to try to make consistent placements across animals, this was difficult because of the large veins on the bulb surface that had to be avoided.

We attempted to confine the recordings to the nerve layer and glomerular layer by inserting the electrode arrays only to the point where the first reliable responses were found on a majority of electrode sites. The depths from the pial surface were measured for 7 of the 11 experiments. These measurements were probably overestimates because of tissue dimpling, but only two arrays penetrated to a nominal depth of more than 800 micrometers.

The activity on adjacent poles of the arrays was clearly related, but it was not identical (see figure 2C). Multiunit activity was evaluated by computing the mean and standard of the voltage on each pole during the one second period before each stimulus. All transients that exceeded 1.5 standard deviations above or below the mean were counted at the point of criterion crossing. (Thus each spike was counted only once). These counts were then low-pass filtered with a function that decayed to half maximum in 50 msec to give smooth estimates of multiunit spiking. These estimates were summed across the poles of the electrode array to help reduce the bias of a single glomerulus or cell. In this summation, we excluded any pole for which there was spike suppression during an orthonasal odor pulse. This exclusion was intended to bias against cells that were influenced by inhibitory interneurons in the bulb.

Not all recording sites gave obvious responses to all odorants. We adopted an automatic criterion for response. The mean summed activity at the peak time was compared to the mean activity in the second before stimulus onset with a t-test using the standard deviation at the time and the maximum standard deviation at any time during the prestimulus activity. The t-test was required to achieve a probability level of $p<.05$. We cannot claim that this is an accurate estimate of the probability, but it reliably picked responses that looked consistent by eye.

Results

Form of the EOG responses

Examples of dorsal and lateral responses to a very hydrophilic odorant (methyl benzoate), a very hydrophobic odorant (vinyl cyclohexane) and one of intermediate solubility (isoamyl acetate) appear in figure 3. The orthonasal responses (Fig. 3A) were similar to our previous reports (Scott et al., 1996;2006). The retronasal responses (Fig. 3B) were very small for methyl benzoate on either site. For vinyl cyclohexane, the responses at both sites were nearly as large as the orthonasal responses. Isoamyl acetate retronasal responses were small during the stimulus presentation, but they terminated with an additional inflection when inspiration occurred after the flow in the expiratory direction. We termed this inflection the “retronasal inspiration” response. In the five animals with dual dorsal electrodes, the early part of the retronasal response to isoamyl acetate was always smaller on the caudal site than the rostral site (Fig. 3C, $p<.01$ by the Wilcoxson rank sum test), but the inspiration responses were not different between the two sites. This inflection disappeared if there was no inspiration, although there could be a slight upward drift that might have resulted from odor diffusion (Fig. 3D). We believe that the inspiration response results from odorant remaining in the dead space in the vestibule of the nose. This odorant is drawn back into the olfactory region by the inspiration. There were no systematic response differences between the two electrodes in the lateral recess.

Relation of EOG to odorant solubility

Quantification of the response peaks across a series of odorants arrayed in the order of their solubility is shown in figure 4 for the first 5 animals. The orthonasal responses were similar to our previous reports in being large dorsally for hydrophilic odorants and large laterally for

hydrophobic odorants (Fig. 4A). The retronasal responses to hydrophilic odorants were reduced relative to orthonasal responses (Fig. 4B). For both the dorsal and the lateral sites, there was a significant variation across odorants for the difference between the orthonasal and retronasal responses ($p<.001$ for the dorsal sites and $p<.01$ for the lateral sites, by the Friedman two-way analysis of variance). At the dorsal site, the retronasal responses were smaller to all odorants except vinyl cyclohexane and heptane ($p<.01$ by the Wilcoxon test). At the lateral site, only anisole, isoamyl acetate, and limonene had significantly smaller retronasal responses ($p<.01$). The mean orthonasal-retronasal differences across odorants correlated strongly with the Hansen solubility parameter for the dorsal site ($r = 0.89$, $p<.01$). The same result was found for calculated odorant polarity. This correlation was not significant ($r =0.19$ for the Hansen parameter) for the lateral site. In contrast, the molecular weight or calculated molecular volume of the odorants did not correlate significantly with the difference in responses at either site.

Two odorants of intermediate solubility (anisole and isoamyl acetate) evoked consistent responses during inspiration after expiration, as illustrated for isoamyl acetate in figure 3. These are quantified with an expanded vertical scale in figure 4C. These responses were more prominent on the dorsal site, although they sometimes occurred for vinyl cyclohexane on the lateral site

Relationship to solubility independent of concentration

A second experiment tested whether some of the differences in figure 4 resulted from different effective concentrations because of the two-fold difference in the air dilution phase between orthonasal and retronasal presentation. For this experiment, we presented four selected odorants in two concentrations with ten-fold differences in the mineral oil dilution. For methyl benzoate, we added another stimulus concentration 50 times stronger. Figure 5 shows recordings from the second set of animals using the electrodes in the same positions as those in figure 4. (There were no systematic differences between the two electrodes of the dorsal set other than those illustrated in figure 3C and no differences between the two electrodes of the lateral set.) The orthonasal responses were greater for the stronger stimuli, showing that the stimuli were within the dynamic range of the system. Neither concentration was effective at producing large retronasal responses to the more polar, hydrophilic odorants, but both concentrations of vinyl cyclohexane evoked responses nearly as large as the orthonasal presentations on both sites. There were effective retronasal inspiration responses to the higher concentrations of anisole and isoamyl acetate, as well as a modest response to both concentrations of vinyl cyclohexane. Only the strongest concentration of methyl benzoate produced a detectable retronasal inspiration response.

We tested the difference in orthonasal vs. retronasal latencies by comparing the orthonasal vs. retronasal responses at the dorsal-anterior and the lateral-dorsal sites, which were the same for the ten animals, using the response to vinyl cyclohexane because it was large in both conditions. For the dorsal-anterior site, the mean (± sem) latency was 202.5 ± 18 msec for orthonasal stimulation and 1154.5 ± 89 msec for retronasal stimulation. For the lateral-dorsal sites, the mean latency was 227 ±26 msec for orthonasal stimulation and 1301 ± 27 msec for retronasal stimulation. Both comparisons were significant as $p<.001$ by paired t-test. Even if the dead space of the retronasal tube (5 ml) is calculated to add up to 750 msec to the latency, the retronasal latencies remain significantly longer ($p<.001$ by paired t-test).

Effect of humidity

One possible concern about these experiments is that the humidity of air passing through the nasal cavity in the two directions would be very different leading to differences in sorption to the cavity walls. We tested this by varying the humidity in the carrier air (figure 6). Peak responses were normalized to the orthonasal isoamyl acetate response for each condition. The

humid condition was run first. The nose was kept humid between stimuli. The vinyl cyclohexane and heptane concentrations are lower than in previous figures, so the shapes of the profiles are slightly different than in figure 4. Both orthonasal and retronasal responses were essentially unchanged by the presentation in dry air. This indicates that modest differences in the humidity of air entering from the lungs vs. the external environment are not the cause of the differences shown in figures 3-5.

Multiunit responses in the olfactory bulb

Multiunit recordings from the superficial layers of the olfactory bulbs of anesthetized rats with multiple electrode arrays were obtained during orthonasal and retronasal stimulation with similar procedures. We cannot claim that these recordings represent only the input patterns. Most likely they represent a mixture of cells in the vicinity of the glomeruli. We excluded all recordings from any probe that showed suppression of activity during the orthonasal presentation of any of the odorants. This was intended to bias against tufted cells or other cells that were responding extensively to inhibitory inputs. We saw no evidence to suggest that any of the recordings were from mitral cells. Most recording sites showed “hash” like multiunit spiking from which single cells could not be isolated, in spite of the fact that we have often recorded large mitral cell spikes with these electrodes. The data shown are for the sums of multiunit activity on the dorsal and lateral arrays as described in the methods. Therefore, even the presence of a large spike on one of the recording sites did not dominate the record.

Figure 7 shows a summed multiunit example from one animal of responses recorded simultaneously from a dorsal and a lateral site on the olfactory bulb. The orthonasal responses were similar to the EOGs in their sharp onset. These orthonasal multiunit responses usually had a sharp initial transient followed by a slower decline in response that was similar to the orthonasal EOGs. This declining phase often ended with a sharp offset (methyl benzoate and isoamyl acetate responses in figure 7). Although the orthonasal multiunit responses were larger at the dorsal site for the polar odorant and larger at the lateral site for the non-polar odorant, these responses also agreed with the EOGs in showing some response to both type of odorant at both sites. However, many of the multiunit recordings did show greater odor specificity than the EOGs, in that they failed to show consistent responses to one or more of the odorants in the series. The retronasal multiunit responses were also similar to the retronasal EOGs. They showed longer latencies from odor onset and a slower rise time. Like the retronasal EOGs the retronasal multiunit responses generally had a more rapid decline than the orthonasal multiunit responses. Two of the multiunit recording sessions also showed a response to inspiration after expiration like those of the EOG responses to isoamyl acetate and anisole. The circled response seen in the retronasal response to isoamyl acetate occurred in each of the individual summed responses to six presentations of the odorant and did not appear in the orthonasal responses.

A summary of the summed multiunit recordings from 11 animals appears in figure 8. Eleven other animals were excluded because the electrodes moved; they failed to show consistent responses due to leaks in the system, or had large responses to the blank, indicating odorant contamination. As explained in the methods, this figure is based on recordings for which the orthonasal responses were reached the t-test criterion of $p<.05$ level. There was greater specificity in the multiunit responses than in the EOGs. The number of odorants failing to evoke significant responses on a site ranged from zero to four. No odorant failed to evoke responses on at least one of the two sites. Even though we used a higher concentration of isoamyl acetate, we did not see significant responses to this odorant at all sites. Therefore, the strategy of normalization to isoamyl acetate was not satisfactory. Instead, we normalized responses to the mean of significant responses to all odorants.

The pattern of responses to orthonasal stimulation was in line with expectations from our previous EOG recordings (Scott et al., 2000) and with the bulb literature (Ho et al., 2006ab;

Takahashi et al., 2004) in showing larger lateral responses to non-polar odorants. The difference between dorsal and lateral responses across odorant was significant ($p<.001$, by analysis of variance). This indicates that our recordings were from parts of the bulb receiving inputs from different types of odorants.

Responses to all odorants were smaller with the retronasal presentation, but the decrease of retronasal responses relative to orthonasal responses was greatest for the more hydrophilic odorants. The difference across odorants between orthonasal vs. retronasal responses was significant ($p<.001$, by analysis of variance) for the dorsal sites, but not for the lateral sites. Thus in spite of any uncertainty about the identity of recorded cells, there is a strong difference in these population responses between the two stimulus modes. This is in line with the EOG results of figures 4, 5, and 6, which show large changes for the polar odorants that activate dorsal epithelium and little effect on the response to non-polar odorants that activate lateral epithelium.

We also analyzed the total data set, including responses that were not judged significant. Analysis of variance was not appropriate because the small non-significant responses made the data deviate strongly from normality. However, the Friedman two-way analysis of variance showed a significant variation across odorants between the two electrodes ($p<.001$) and showed a significant variation across odorants for difference between the orthonasal and retronasal responses at the dorsal sites ($p<.01$). This indicates that the data analysis was not biased by selection of large responses.

Discussion

Responses are different in the two modes

These data show that odorant responses differ between the orthonasal and retronasal modes. The EOGs are a direct measure of the receptor cell population response, thus providing the best estimate of which odorants reach the receptors and of their relative concentrations. Our results are complementary to the results of Frasnelli et al. (2005) and of Heilmann and Hummel (2004), who measured odorant concentrations near the olfactory cleft. Those studies had the advantage of using human subjects, but the disadvantage that tubes placed in the nose to sample air could disturb the air flow in the nose. In addition, that technique does not measure the time course of odorant arrival or the concentrations that partition into the mucus layer surrounding the receptor cilia. Within our sample, non-polar, hydrophobic odorants are more effective than hydrophilic odorants in retronasal stimulation of olfactory receptors. The result is independent of odorant concentration and of carrier air humidity.

We also found longer latencies for the retronasal responses suggesting that the flow of air entering the olfactory region takes longer, even when the total flow rate in the nasal cavity was the same. These latencies are not accounted for by the dead space of the stimulus delivery system. They may be related to the dead space in the nasal cavity, but that is difficult to estimate. These differences suggest that a sorptive mechanism produces these response differences by removing the polar odorants from the air stream before it reaches the olfactory receptor cells. This might occur either by more internal dead space with retronasal or because only a fraction of expired air enters the olfactory region at slower rate.

The form and interpretation of responses

While the ultimate goal of any physiological investigation of air flow in olfaction is to understand the effects produced in normal breathing, sniffing, chewing and swallowing, this study has begun that investigation with an artificial situation that allows reasonable control of concentration and air flow. The durations of our stimuli were single unphysiologically long

pulses, but previous work has shown that it can take at least one second for some responses to reach a peak with orthonasal flow (Scott et al., 2006). We found that under those conditions of long duration stimulation, the absolute and relative sizes of most odorant responses were similar to those with direct application odorant to the exposed epithelium (Scott-Johnson et al., 2000; Scott et al., 2006). The current data set a kind of upper limit and tell us that with long duration stimuli very non-polar odorants can approach complete access to the epithelium in the retronasal mode. In contrast, very polar odorants had very depressed retronasal responses even with very long durations. The long duration, single stimuli also allowed demonstration of response latencies, which would not have be clear with multiple pulses or studies in behaving animals.

The EOG is a useful measure of response in the epithelium for this study because of the relative ease of recording and the fact that it represents a population of receptors. It gives a more accurate measure of the amount of odorant reaching the receptors than any attempt to measure odorant concentration in the nasal cavity because of the small spaces in the olfactory region and the necessity for odors to cross the air-liquid interface to reach the receptor sites. The major conclusion that retronasal presentation produced decreased response to polar odorants and longer latencies for all odorants was confirmed in multiunit recordings from the superficial layers of the olfactory bulb. The multiunit bulb recordings differed from the EOGs in one important respect. The bulb responses were more specific, so that in some preparations there were strong responses to some odorants of a class (either polar or non-polar) and weak responses to others. This was not the case for the EOGs where all polar odorants produced significant responses in the dorsal epithelium and all non-polar odorants produced significant responses in the lateral epithelium. For that reason, the EOG is a better method for characterizing the overall chemical properties correlating with differences between orthonasal and retronasal responses, although it will ultimately be very important to know the effects of these differences on the responses of olfactory bulb mitral/tufted cells.

The multiunit records add the important information that the difference between orthonasal and retronasal responses is preserved at the upper layers of the bulb. The summation across the multiprobe arrays and the numbers of preparations tested makes it clear that the results are not dominated by a single cell type. Because of this exclusion and the shallow depth of the electrodes, very few, if any, of the recordings are likely to have been from mitral cell. Excitation is spread from external tufted cells to short axon cells and then to inhibitory neurons in the glomerular layer (Aungst et al., 2003). Thus inhibition can be induced without involvement of the granule cell population. Our intent in restricting the recordings to apparent excitatory responses was to minimize the canceling out of excitatory responses by inhibitory interactions. The electrode array placements were not exactly the same in all animals because we had to avoid the large surface veins. In addition, the potential for spread of excitation in the glomerular layer (Aungst et al., 2003) makes it likely that these recordings do not represent only local inputs. However, the pattern of response to polar vs. non-polar odorants in the orthonasal recordings confirms that the dorsal and lateral arrays were in appropriate positions to reflect the differences between the dorsal and lateral parts of the epithelium. This spatial distribution of sensitivity in the bulb has been reported in a number of investigations (Imamura et al., 1992; Ho et al., 2006ab; Takahashi et al., 2004), and it agrees with our observations in the epithelium (Scott and Brierley, 1999). The multiunit records also confirmed the longer latencies and slower rise times of the EOG results. This suggests a slower access of odorants to the receptor neurons for retronasal presentation. As we discuss below this temporal difference is an important clue to the mechanism of the orthonasal/retronasal response differences.

The responses at the end of the retronasal pulse for some odorants are probably due to the inspiration of odorants remaining in the anterior part of the airway. This is supported by the disappearance of those responses if the stimulus is terminated without inspiration. This effect

in the EOG shows the differential effects on odorant access even within the dorsomedial recess of the nasal cavity. The inspiration responses after retronasal stimulation were most prominent for odorants of intermediate solubility. Here those odorants were isoamyl acetate and anisole, but we have seen similar results for 2-hexanone (Phan, unpublished data). Only a subset of flavor related odorants are in this solubility range. Odorants are known to enter the nose during chewing and swallowing (Burdach and Doty, 1987; Hodgson et al., 2003). These movements and ongoing respiration probably facilitate the movement of odorants into the olfactory region. It may be that the inspiration responses are important in this context. These retronasal inspiratory responses were confirmed in two of the bulb recordings, but they were less prominent in the bulb recordings. Only future experiments would determine whether these responses are significant in control of bulb activity. It would be important to determine whether human observers can discriminate the sensations associated with these two aspects of retronasal olfaction.

The mechanism of differences

The reduced response to hydrophilic odorants during retronasal presentation must result from reduced concentration in the space directly overlying the receptor neurons because there were strong orthonasal responses to these stimuli before and after the retronasal tests. The size of the EOG is monotonically related to odorant concentration (Scott et al., 2000; 2006) and the response seen with orthonasal stimulation of long duration is approximately equal to that seen when odorants are directly applied to the headspace over the epithelium (Scott-Johnson et al., 2000; Scott et al., 2006). Thus it appears that with sufficiently long odor pulses at high flow rates even rather hydrophilic odorants will reach the receptor sheet in high concentrations in the orthonasal mode, but the concentrations of hydrophilic and moderately soluble odorants are very depressed in the retronasal mode. It is likely that the rate of air movement is the mechanism for this differential effect. The modeling of the rat nasal cavity by Zhao et al. (2006) suggests that expiratory air streams do not enter the olfactory region as effectively as the inspiratory streams. A reduced mass movement of air would provide more time for sorption of polar, hydrophilic odorants. On the other hand a slower transfer of air into the olfactory region is still consistent with strong responses to non-polar, hydrophobic odorants (Mozell et al., 1991; Scott et al., 2006). Unfortunately, direct measurements of the air flow in the small spaces of the rodent nasal cavity are not available, but these results are very consistent with the predictions of responses in the rat from the air flow model of Zhao et al. (2006).

The correlation of solubility with the orthonasal/retronasal difference is consistent with the speculations by previous investigators of human retronasal olfaction (Pierce and Halpern, 1996; Small et al., 2005; Sun and Halpern, 2005) that the sorption of odorants is a significant factor in the differences between orthonasal and retronasal odorant recognition. Most human psychophysical investigations have used complex odor stimuli to simplify recognition by subjects. We have followed the strategy of using pure odorants with a range of solubilities in order to test the sorption hypothesis. In such cases, the pattern of access to the olfactory region by the various odorous components could provide one cue by which the sensations differ. As in our recent publications (Scott, 2006; Scott et al., 2006), we have represented odorants by the Hansen solubility parameter. This descriptor is one of many that might be applied to the current data to show the same relationship. We found it useful because it is easily calculated, and the retention time information used by Mozell et al. (1991) is not available for all of the odorants that we have employed. There are other mechanisms such as odorant binding proteins (Löbel et al., 2002; Hajjar et al., 2006) and enzymes that break down odorants (Leclerc et al., 2002) that could influence the concentration of odorants in the airway. At this point, we know too little of those mechanisms to propose clear tests of those alternatives.

Alternatively, some of the response differences among these odorants could be due to other mechanisms such as diffusion (Zhao et al., 2004; 2006). The substantially longer latencies of the retronasal responses or the relatively smaller retronasal response to limonene, a larger molecule than heptane or vinyl cyclohexane, might support a diffusion explanation. Calculated molecular volume, which should be a factor in diffusion, was not correlated with the orthonasal-retronasal difference. Investigations with a larger, even more diverse sample of odorants would be necessary to test more complicated relationships between stimulus properties and the behavior in this system.

Functional implications

Our results are consistent with the speculations by previous investigators of human retronasal olfaction (Pierce and Halpern, 1996; Small et al., 2005; Sun and Halpern, 2005). It is complementary to the results of Frasnelli et al. (2005) and of Heilmann and Hummel (2004). While they were able to measure odorant concentrations near the olfactory cleft, they not measure receptor responses. Thus they were unable to know concentrations in the narrow space of the olfactory cleft or to know how effectively the odorants traversed the mucus to reach receptors.

In complex odor blends, the pattern of access to the olfactory region by the various odorous components could provide one cue by which the sensations differ. Psychophysical studies (Sun and Halpern, 2005) and imaging of cortex/basal ganglia have been used to study higher order response to stimuli presented in the orthonasal vs. retronasal modes. It is not yet known whether the circuitry of the olfactory bulb compensates for the mode of stimulation under control of cues like trigeminal innervation. It seems likely that the bulbar output cells show very different responses to mixtures in the two stimulus modes. Non-polar odorants would likely partition more effectively into dietary fat. The enhanced response to non-polar odorants in fatty foods may be one element of preference for fat in the diet.

These results do not diminish the important of taste or other cues in retronasal olfaction. One possible cue is trigeminal stimulation. Frasnelli et al. (2004) studied event related potentials and found different cortical reactions depending on whether a trigeminal stimulus (carbon dioxide) was applied to the anterior or posterior part of the nasal cavity. Another contributing factor could be the presence of neural activity that codes the sniff and tells the brain about the direction of air flow. An example of this would be the activity correlated with sniffing and mouth movements in motor regions of the brain (Burdach and Doty, 1987; Small et al., 2005; Sobel et al., 1998).

There is growing recognition of the importance of retronasal olfaction for normal oral sensation. In addition to psychophysical and imaging approaches, direct measurement of aroma release by materials in the mouth (Mestres et al., 2005) and measurement of intranasal concentrations in the human mouth (Frasnelli et al., 2005) are being employed in these studies. Observations like these on the olfactory epithelium can be used to investigate the chemical parameters of retronasal olfaction with sufficient temporal resolution to distinguish the inspiratory and expiratory phases. The rat seems an appropriate model for further investigation because of behavioral reports indicating the importance of retronasal olfactory for the rat (Bouton et al., 1986; Inui et al., 2006; Slotnick et al., 1997) as well as the presence of flow modeling for both species. The animal model has the advantage of being applicable to a wide range of stimuli and stimulus conditions that would facilitate further investigation of peripheral and central mechanisms.

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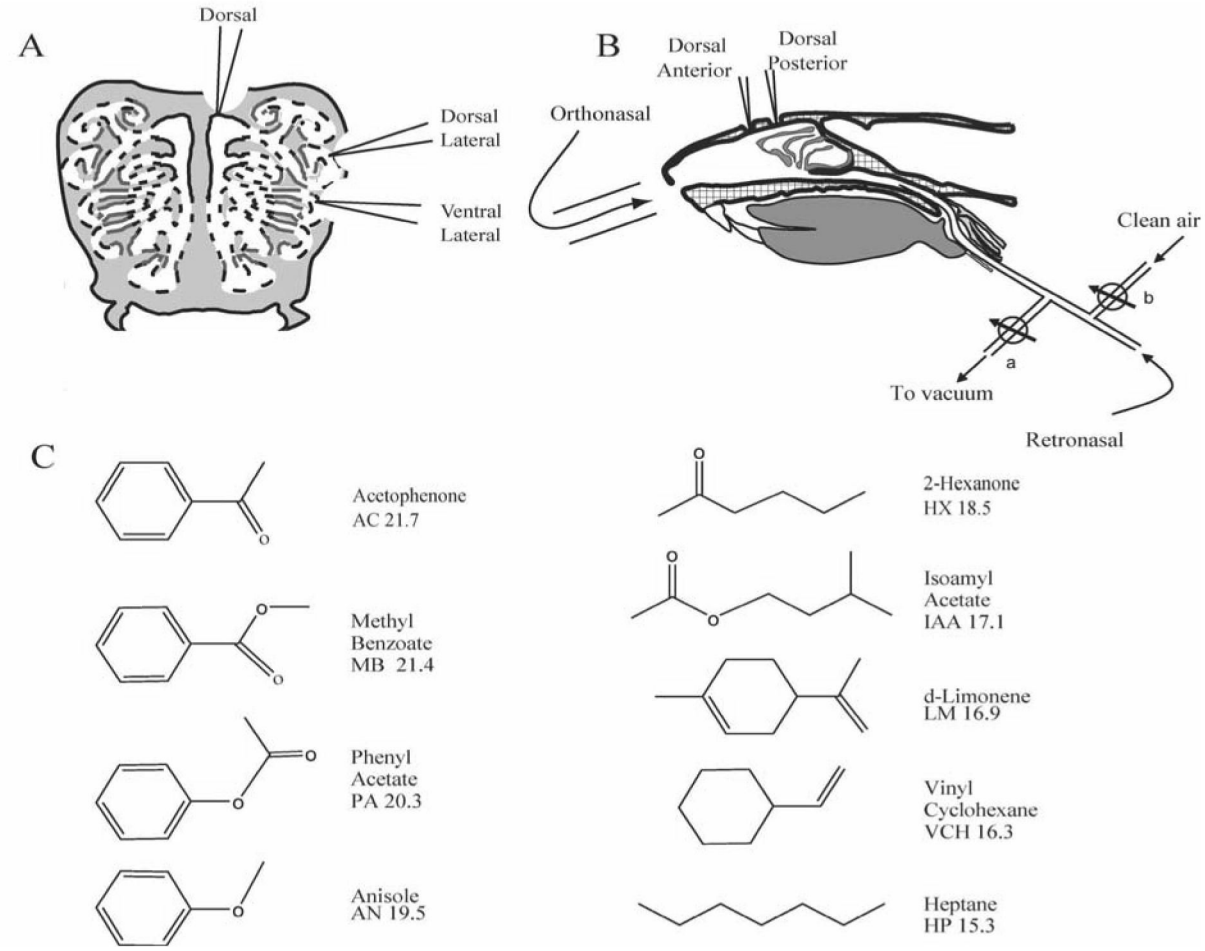

Figure 1.

The experimental setup and odorants used in this study. Panel “A” shows a schematic cross section of the rat nose to illustrate the position of dorsal and lateral electrodes relative to the gene expression zones described in the literature. Panel “B” is a schematic midline view showing the position of the two dorsal electrodes. Panel “B” also shows how the retronasal cannula was set up. Clean air was flushed through the retronasal cannula while odorant concentration was building up. Valves “a” and “b” were closed at the end of odorant buildup to force air through the nose with a sharp temporal onset. Panel “C” shows the odorants used in these experiments along with the abbreviations used in the figures and the Hansen solubility parameter described in the methods.

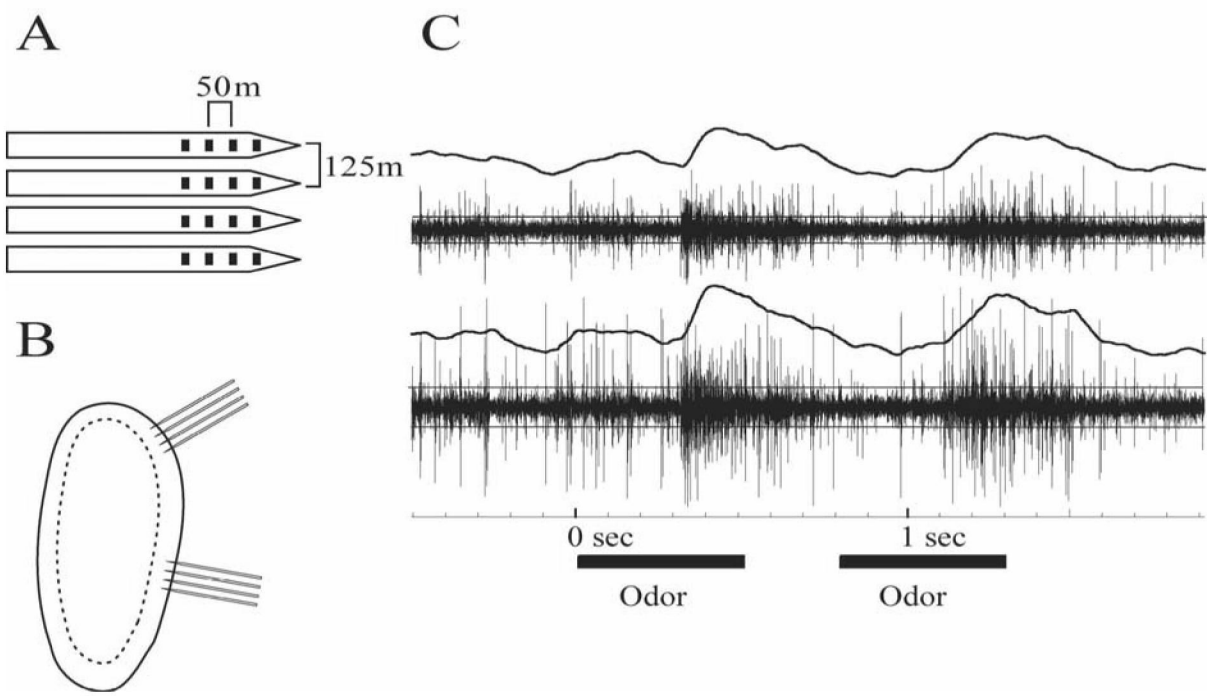

Figure 2.

illustrates simultaneous recordings two poles of an electrode array located in the superficial layers of the lateral part of the olfactory bulb and the data analysis. Electrode arrays (A) were driven into the dorsal and lateral aspects of the bulb (B) until multiunit responses were found to several of the odorants in the sample series. Only rarely were apparent mitral cells (judged by spike size and response pattern in this case) encountered. All points where the voltage records crossed the value 1.5 standard deviations ± the mean voltage (thin lines) in the one second interval before stimulation were marked. These were low-pass filtered with a function that decayed to half maximum in 50 msec to give a record like the ones shown above in black. Note that large spikes are not weighted more than small spikes. Even though these two adjacent traces had different spikes, the summed records were very similar. The heavy bars under the traces show the two odor pulses used in this recording. This figure illustrates the response to a pair of odor pulses to make the odor response more obvious, although the subsequent figures show responses to longer, single odor pulses.

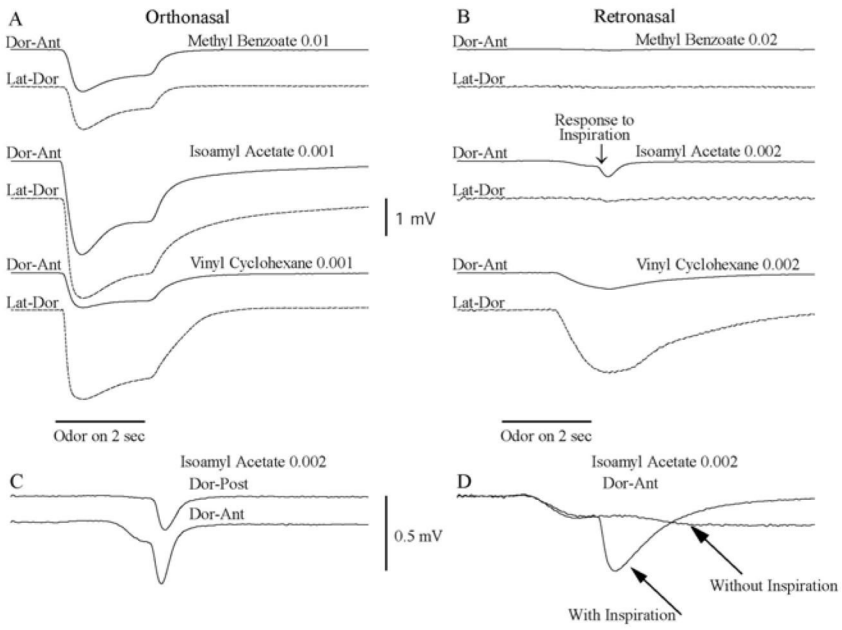

Figure 3.

Panel “A” shows sample traces to three odorants presented orthonasally. There was a substantial response to each odorant at a latency of about 100 msec. Panel “B” shows responses in the same animal to retronasal presentation of the odorants. The concentrations are greater because of the differences in the air dilution procedure in the two stimulus modes (see methods). There was little response to the most soluble odorant (methyl benzoate) at either site. Isoamyl acetate produced a small response on the dorsal electrode during the odor presentation followed by a distinct, short response shortly after cessation of the odor pulse (indicated by arrow marked “Resp to Insp”). The hydrophobic odorant (vinyl cyclohexane) evoked strong responses that differed in latency and time course from the orthonasal responses. All traces are means of three responses on the dorsal anterior and dorsal lateral electrodes shown in figure 1. Panel “C” compares simultaneous recordings from the Dorsal Anterior and Dorsal Posterior during the retronasal condition. In general the Dorsal Anterior and Dorsal Posterior responses were qualitatively similar except for the larger retronasal inspiratory response on the Dorsal Posterior electrodes (see text). Panel “D” shows an example of the retronasal isoamyl acetate response from another animal. In this case, the retronasal response was larger than in panel “B” and the latency was shorter. The traces show overlays of recordings in which the retronasal stimulus was terminated with the 500 ml/min inspiration evoking additional response (as in “B”), or in which the odor flow was just stopped without an inspiration. The calibration for panel “D” is for the trace with inspiration. The other trace is adjusted in size to overlap the initial portion. Panels “A” and “B” have a common voltage calibration. Panels “C” and “D” have a common voltage calibration. The time/odor stimulus bars apply to all traces.

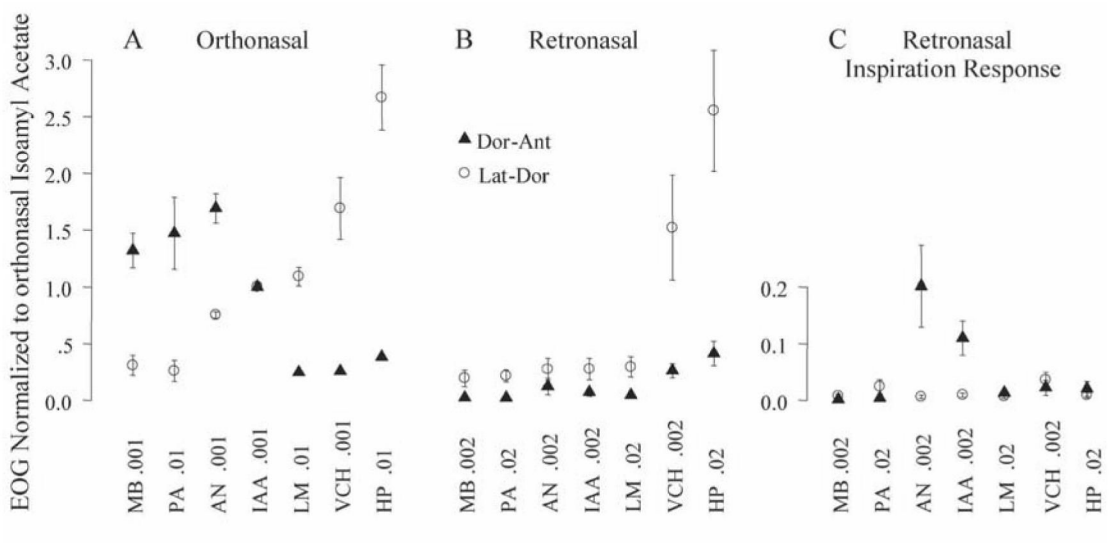

Figure 4.

Panel “A” shows the peak responses to a series of 7 odorants normalized to the response to isoamyl acetate (mean ± sem, n=5 animals). This experiment used only the Dorsal Anterior and Lateral Dorsal electrodes of figure 1. The odorants are arrayed in the order of the Hansen solubility parameter with the most soluble at the left. The abbreviations and values of the Hansen parameter are given in figure 1. The relative size of responses at the two sites changes with the solubility parameter. Panel “B” shows the peak response during the 2 second expiratory phase of the retronasal stimulus. The responses on both sites are smaller for the odorants with higher Hansen solubility parameters. Panel “C” shows the peak size of the inspiration retronasal response. It is large only for the odorants of intermediate solubility. This panel is shown at five times the amplification of panels “A” and “B”.

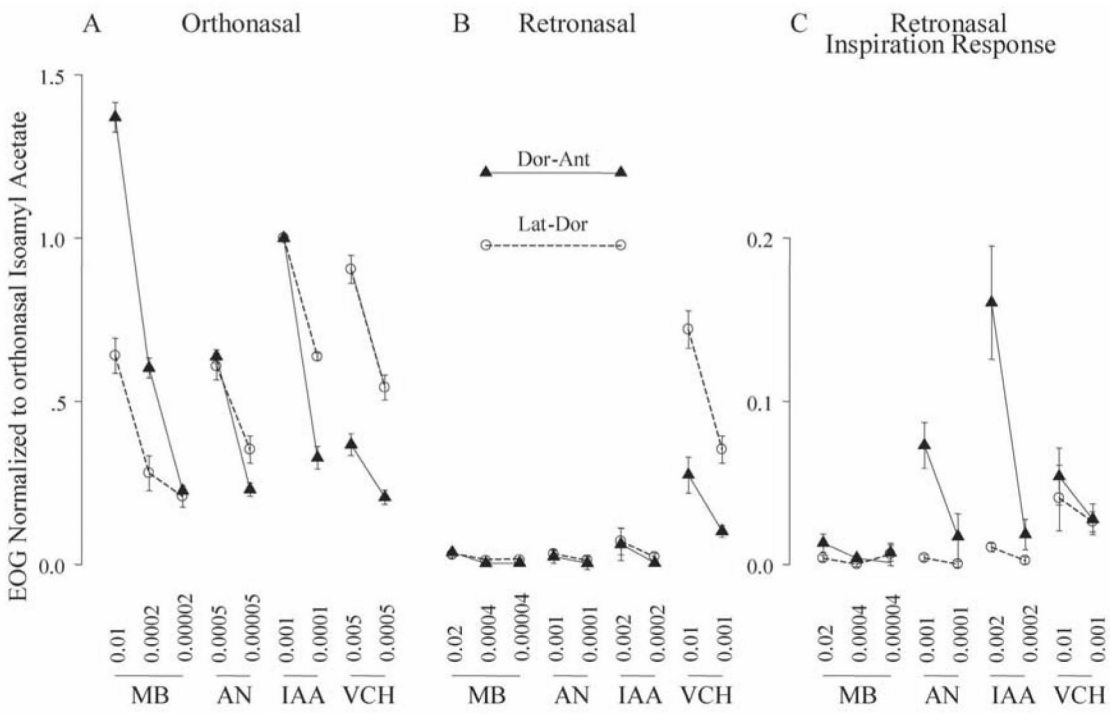

Figure 5.

shows the mean ± sem (n= 5 animals) for responses to four odorants at two or more concentrations for the Dorsal Anterior and Lateral Dorsal electrodes of figure 1. Odorants with higher Hansen parameters are to the left of each panel. The responses on the other electrodes were qualitatively similar except as illustrated in figure 2C and described in the text.

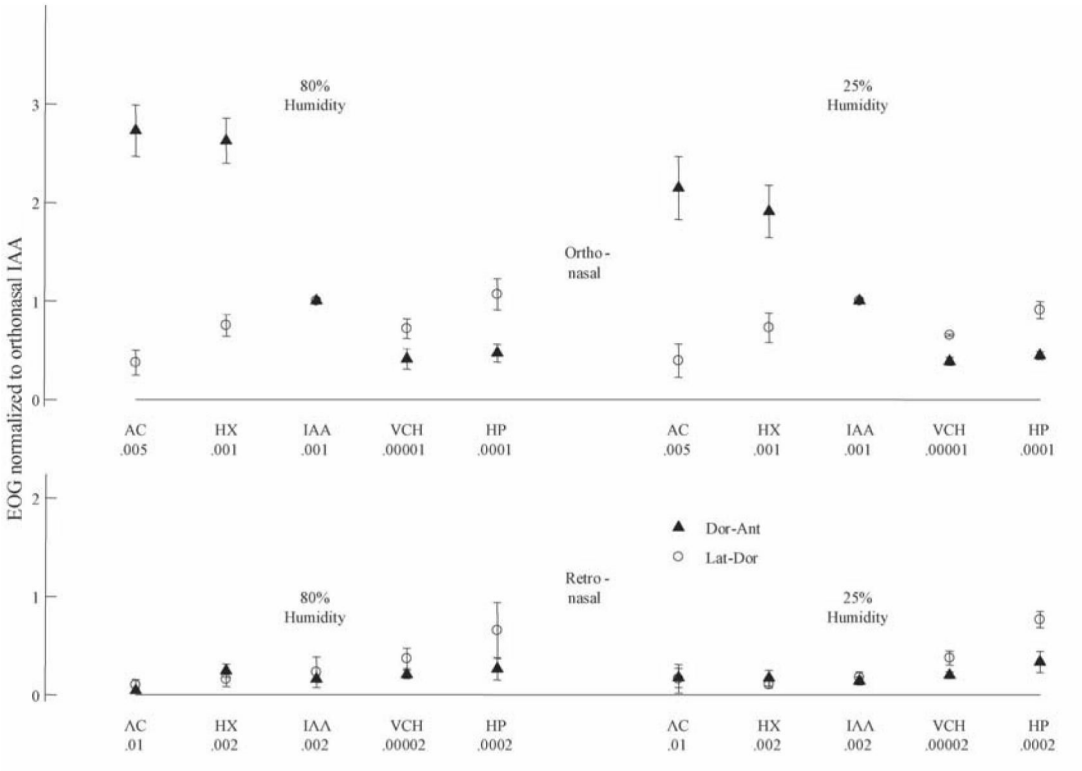

Figure 6.

The relative humidity in the carrier air did not affect the pattern of mean (± sem) responses to orthonasal vs. retronasal stimulation. This figure includes two odorants (AC=acetophenone & HX=2-hexanone) not shown in previous figures. N=4 for all odorants except AC (N=3).

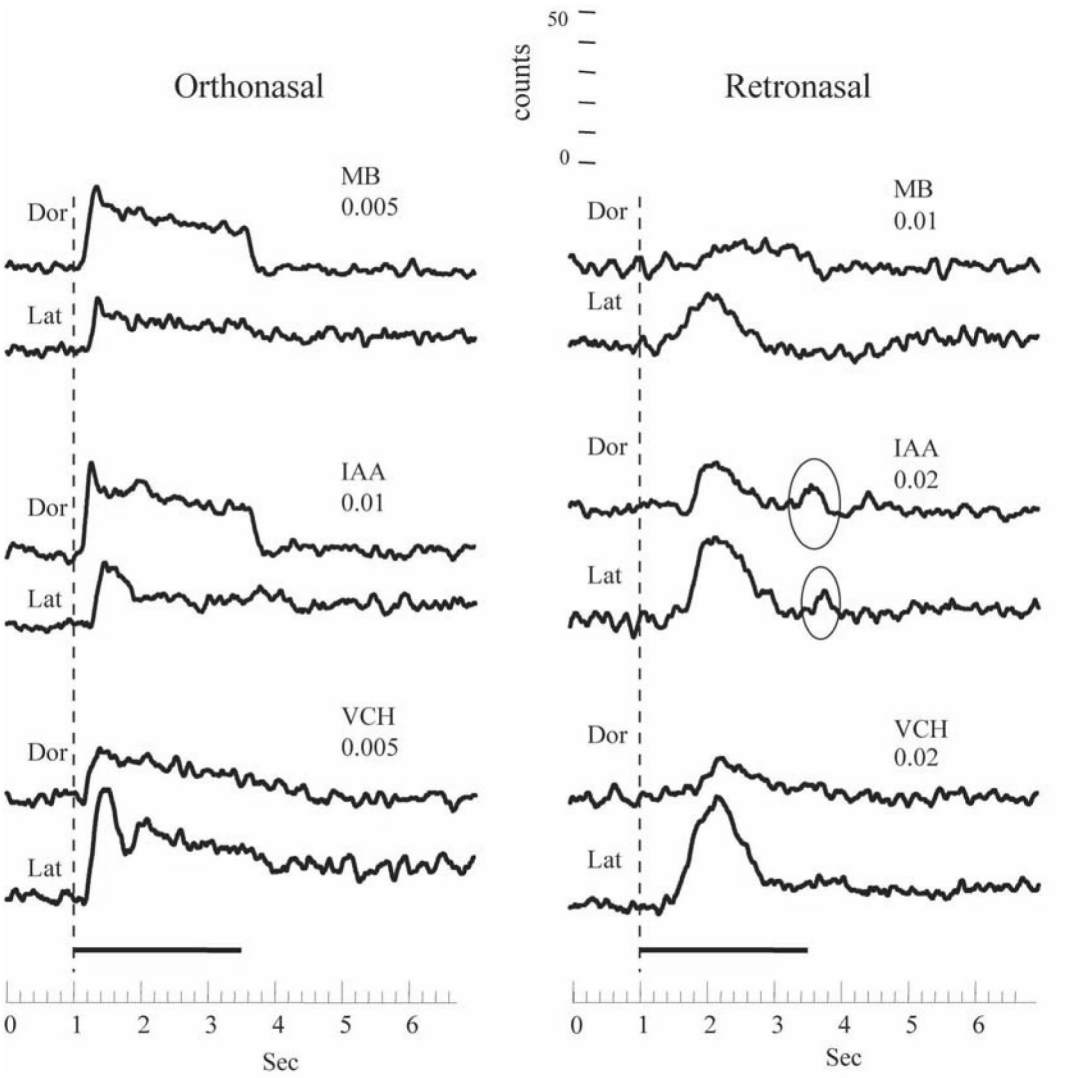

Figure 7.

shows summed responses from the superficial layers of the dorsal and lateral olfactory bulb for 2.5 sec odor pulses (bars below traces). In this animal the lateral responses to these three odorants were slightly reduced for retronasal stimulation. The dorsal responses were more severely reduced and were reduced in proportion to solubility. The orthonasal traces are means for three presentations of each odorant. The retronasal traces are means of nine presentations (six before the orthonasal record and three after the orthonasal records). Note the longer latencies and slower rise times of the retronasal responses and their similarity to the EOGs in figure 2. The circles indicate inspiratory responses like those in figure 2.

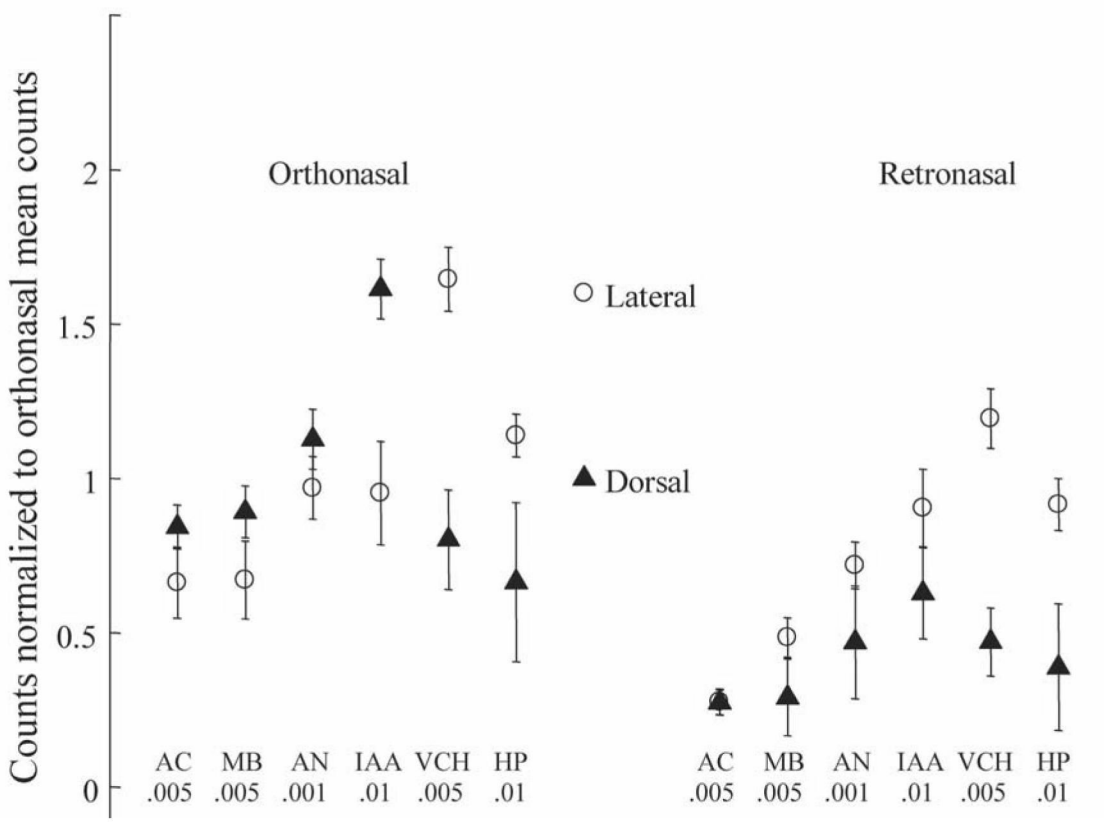

Figure 8.

shows peak multiunit responses (mean ±sem) from nine animals with the odorants arrayed in the order of solubility. This summarizes the significant responses from 11 successful experiments in which the peak counts were normalized to the mean of orthonasal peaks for the six odorants. The difference across odorants for the orthonasal dorsal-lateral responses was significant $p<.001$ by analysis of variance ($F= 7.91$ with 5,36 df). The difference across odorants for the dorsal orthonasal-retronasal responses was significant $p<.01$ by analysis of variance ($F= 4.92$ with 5,48 df).