

EOG Responses in Anesthetized Freely Breathing Rats

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Abstract

In mammals, access of odor molecules to the olfactory receptor neurons is controlled by respiratory activity. Thus, anesthetized, freely breathing rats were used to record from the olfactory mucosa in the intact nasal cavity (electroolfactogram or EOG) so as to study global response characteristics to odor stimuli. During alternation of the inspiratory phases of odor sampling and expiratory phases, the response was a succession of individual EOG events synchronized with respiration. These were characterized by a steep decrease that started ~100–150 ms after the beginning of inhalation, reached its maximum at the transition between inspiration and expiration and was followed by a slower rise until the next inhalation. They were greater during the first respiratory cycles following odor stimulation onset. Thereafter their amplitudes decreased throughout odor delivery, but a significant EOG signal was still present at the end of short (10 s) and long (60 s) odor presentations. Amplitude increased with odor concentration, but much less than expected from concentration changes. Lastly, for some odors EOG responses persisted well beyond the end of stimulation. These results are in agreement with the respiratory synchronization of mitral cell activities observed during short odor presentations and long duration odor exposures. They underline again the importance of taking into account the respiratory activity in studies on the functioning of the olfactory system.

Introduction

In mammals, odor molecules do not have immediate access to the olfactory receptors (ORs) of the olfactory receptor neurons (ORNs). They have to be carried to the entrance of the nostrils by the inspiratory air flow and then traverse the highly complex (Clancy *et al.*, 1993) and sorptive zone of the nasal fossa to be finally absorbed in the mucus layer lining the olfactory epithelium. Their transport to the ORs has been shown to depend on several parameters, such as the anatomy and resistance of the upper nasal pathways (Teghtsoonian and Teghtsoonian, 1984; Hornung *et al.*, 1987b), the air velocity in these pathways (Schneider *et al.*, 1965; Rehn, 1978; Mozell *et al.*, 1984, 1991a,b), the diffusivity of the odorant in air and mucus (Hornung and Mozell, 1977, 1981; Hornung *et al.*, 1987a) and the thickness of the mucus layer (Getchell and Getchell, 1977). For example, polar odorants tend to be removed from the air stream when odors are drawn along the mucosal surface in the frog (Mozell, 1964, 1970; Hornung and Mozell, 1977; Mozell *et al.*, 1984, 1991a,b) and the rat (Kent *et al.*, 1996). The differential adsorption of odor molecules onto the surface of the olfactory epithelium might be a mechanism involved in odor recognition (Mozell, 1964, 1970). An increasing amount of data suggests that the olfactory system might take advantage of this ‘imposed spatial patterning’ (Moulton, 1976) and of the ‘inherent spatial patterning’ due to regional

differences in sensitivity across the olfactory epithelium (Kauer and Moulton, 1974; Moulton, 1976; Thommesen and Doving, 1977; Kubie *et al.*, 1980; Mackay-Sim and Kubie, 1981; Edwards *et al.*, 1988; Kent and Mozell, 1992; Mackay-Sim and Kesteven, 1994; Youngentob *et al.*, 1995; Kent *et al.*, 1996; Scott *et al.*, 1997; Scott and Brierley, 1999) to achieve odor discrimination. It could, for example, utilize this chromatographic-like process to separate the odorants in a mixture in space and time along the incoming flow path (Kent *et al.*, 1996).

In contrast, neither the physiological data reported above nor recent theoretical models of olfaction (Hahn *et al.*, 1994; Keyhani *et al.*, 1997) provide insight into the dynamics of the responsiveness of the olfactory epithelium across successive respiratory cycles in freely breathing animals. Indeed, most of the investigators puffed the odorants directly onto the exposed olfactory mucosa and the others drew odorants artificially through the nasal cavity. Therefore, the goal of the present study was to analyze the characteristics of the responses of the olfactory epithelium in animals where the alternation of inspiratory and expiratory air flows was maintained. This was achieved by recording the signal evoked by different odors in the dorsal aspect of endoturbinates II of anesthetized freely breathing rats. We investigated the characteristics of the successive

electroolfactograms (EOGs) evoked by the respiratory cycles during the stimulation periods and their changes with stimulus characteristics.

Methods

Surgical methods

Experiments were performed on anesthetized freely breathing animals. They were carried out in accordance with the European Communities Council Directive for the care and use of laboratory animals. Adult Wistar rats (weight 250–300 g) were initially anesthetized by an i.p. injection of 3 ml/kg Equithesine (a mixture of pentobarbital sodium and chloral hydrate). Anesthetic was supplemented as necessary to maintain a deep level of anesthesia, as determined by the lack of withdrawal reflex of the leg in response to a moderately intense toe pinch, and the surgical wounds were regularly infiltrated with 2% Procaine. Rectal temperature was maintained at $37 \pm 0.5^\circ\text{C}$ by a homeothermic blanket (Harvard Apparatus, USA) throughout the experiment.

Electrophysiological recordings

EOG recordings were performed at the surface of the dorsal aspect of endoturbinates II with Ringer–agar-filled glass micropipettes of $\sim 50\ \mu\text{m}$ diameter. Access to the olfactory epithelium was gained by drilling a small hole in the nasal bone directly above this turbinate and by using the tip of a sharpened tungsten barrel ($250\ \mu\text{m}$ diameter, $10\ \mu\text{m}$ at the tip) to make a small hole in the exposed epithelium covering the roof of the nasal cavity. The electrode was then gently slipped into this hole, which closed itself up so that no air could enter around the electrode. The electrode was advanced until its tip touched the olfactory epithelium, as shown by deflection of the EOG signal.

The EOG signal was amplified by a conventional amplifier (DC 30 Hz cut-off), displayed on an oscilloscope and recorded on a data tape recorder (Biologic, France) and a CED-1401 data acquisition system (Cambridge Electronic Design Ltd, UK) connected to a computer for off-line analysis. Respiratory activity was simultaneously recorded through a thermistor placed at the tip of the contralateral nostril, amplified and filtered between 0.1 and 30 Hz. The computer routinely sampled signals at 200 Hz.

Odor stimuli

Five reagent-grade chemicals were utilized as stimuli: acetophenone (ACE), cineole (CIN), isoamyl acetate (ISO), *p*-cymene (CYM) and methylamylketone (MAK).

Odorants were delivered using a flow dilution olfactometer described in detail elsewhere (Vigouroux and Chaput, 1988). Briefly, the nozzle of the olfactometer was continuously supplied with a main flow of pure, humidified air (28 l/min). A second flow of pure air (2 l/min) was injected into this flow between odor delivery periods and was

Table 1 Full and abbreviated names of the five pure odorants, saturated vapor pressures (SVP) at 25°C (from the *Handbook of Chemistry and Physics*, 76th edn), concentrations in the saturated vapor phase of these odorants and dilutions performed in terms of proportions of SVP so as to obtain standard concentrations of the different odorants at $\sim 1.2 \times 10^{-7}$ mol/l (corresponding to a partial pressure of 2.9 Pa)

Odor	Saturated vapor pressure (SVP; in Pa)	Concentration (mol/l) in the SVP	Dilution (proportion of SVP)
Acetophenone	49	1.98×10^{-5}	6.0×10^{-2}
Cineole	260	1.05×10^{-4}	1.1×10^{-2}
Isoamyl acetate	728	2.94×10^{-4}	4.0×10^{-3}
<i>p</i> -Cymene	190	7.68×10^{-5}	1.5×10^{-2}
Methylamylketone	490	1.98×10^{-4}	6.0×10^{-3}

replaced during stimulation by an equivalent flow of odorized air. This odorized flow was obtained by pumping a predetermined proportion of saturated vapor from 50 l Tedlar bags connected to the olfactometer through pre-adjusted interchangeable needle valves. The pump started 10–15 s before odor delivery so as to allow odor concentration to stabilize in the line and was exhausted until stimulation onset.

In this study, odorants were delivered at the same vapor phase in order to compare their EOG responses. They were diluted differentially to achieve a standard vapor concentration of $\sim 1.2 \times 10^{-7}$ mol/l (Table 1). These standard concentrations were delivered first. They were considered high enough to recruit most of the ORNs responding to the delivered stimulus. Other concentrations were multiples of these standard concentrations.

Stimuli were delivered at intervals of at least 2 min during short (10 s) and long duration (20, 30, 40 and 60 s) odor presentations. In both cases, stimulation onset was initiated 10 ms after the beginning of an expiration, so that the first inspiration included in the stimulation corresponded to a complete stimulation period. Short duration presentations of the odorants delivered in the order ACE, CIN, ISO, CYM and MAK at their standard concentration and at twice this standard concentration were first performed. Then increasing concentrations were delivered. Lastly, long duration odor presentations were performed.

Data analysis

Figure 1 shows representative examples of the EOG signal and respiratory activity recorded in freely breathing animals. The EOG signal (top trace in each pair) is composed of a succession of negative deflections synchronized on the respiratory activity (bottom trace), followed by a return to or above the spontaneous baseline. It was processed to determine the amplitudes of the individual EOGs and the amplitude and position of the largest EOG in

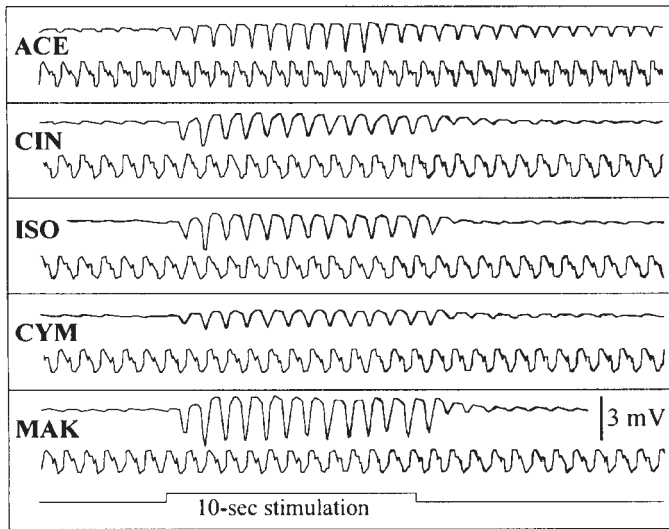


Figure 1 The pairs of traces show the EOG signal (top) and the respiratory activity (bottom) recorded before, during and after 10 s presentations of the five odorants delivered at their standard concentrations. The negative deflections on the respiratory traces correspond to inspiration and the positive ones to expiration. The scale applies to all EOG traces.

each set (maximum EOG). Individual EOG amplitudes were obtained by subtracting the mean amplitude of the fluctuations of the EOG signal during the 30 respiratory cycles preceding each stimulation from the measured EOG amplitudes and by comparing them with the deflection elicited by a known calibration voltage.

Individual EOG amplitudes were also measured after stimulation cessation to analyze the persistence of the signal. A significant EOG signal was said to persist as long as the individual EOG amplitudes were greater than the mean ± 2 SD of the amplitudes of the EOG signal in the 30 respiratory cycles recorded before each stimulation.

Results

EOG examples

Figure 1 shows representative examples of the EOG responses to the five odors delivered at their standard concentrations to the 27 male rats included in this study. The EOG signal recorded in freely breathing animals was composed of a succession of negative slow potentials synchronized with the respiratory rhythm. These potentials always appeared within the first inspiration following stimulation onset. They were characterized by a steep decreasing phase followed by a slower rise. They began ~ 100 – 150 ms after the beginning of inhalation, reached their minimum at the transition between inspiration and expiration and terminated when the expiratory flow stopped. Generally, EOGs of greater amplitude occurred in the first respiratory cycles following stimulation onset. As

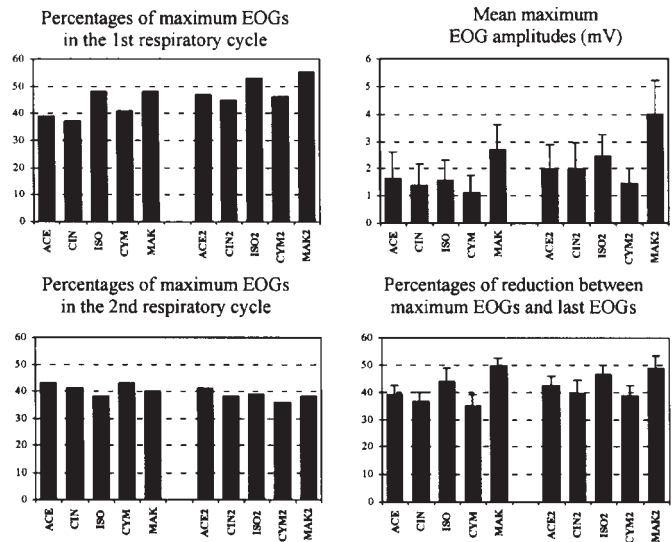


Figure 2 Proportions of maximum EOGs in the first and second respiratory cycle (top and bottom left-hand histograms, respectively) of 10 s presentations of the five odorants at their standard concentrations (first five bars) and at two times their standard concentrations (ACE2 to MAK2). Mean maximum EOG amplitudes (top right histogram) and mean percentage reductions between the maximum and last EOGs (bottom right). SD calculations were performed on individual percentages.

shown later, they disappeared rather rapidly after the end of odor delivery, except for ACE.

Characteristics of the EOGs

EOG characteristics were determined using the greatest EOG in the stimulation period (maximum EOG) as a reference. The upper and lower left-hand histograms in Figure 2 show the proportions of trials in which the maximum EOG occurred during the first or second respiratory cycle following stimulation onset, respectively. At standard odorant concentrations, $\sim 40\%$ of maximum EOGs occurred during the first or second respiratory cycle. Doubling concentrations (bars ACE2 to MAK2) did not produce a significant change in the proportion of maximum EOGs observed in the first cycle for any of the odorants (Wilcoxon matched pairs signed ranks test, $P > 0.05$).

MAK induced the greatest maximum EOGs among the five odorants delivered at the standard concentration (upper right-hand diagram in Figure 2). ACE, CIN and ISO produced significantly smaller responses and CYM evoked the smallest responses. Lastly, maximum EOG mean amplitudes were increased, but not doubled, when the concentration was doubled.

Individual EOG amplitudes decreased over the course of stimulation for all odorants, as shown by the mean percentage reductions from the maximum EOG to the last EOG of the stimulation period (designated as last EOG in the lower left-hand histogram in Figure 2). Similar percentage reductions were observed for double concentrations and the

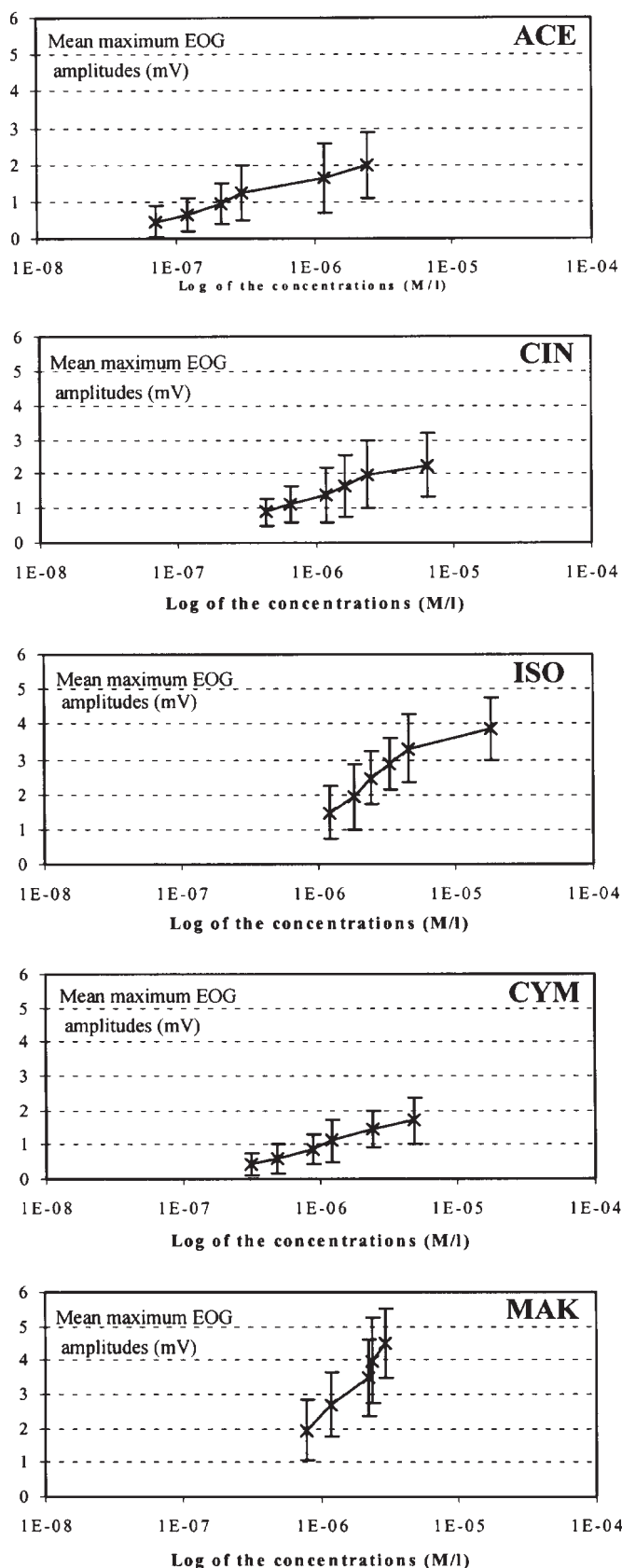


Figure 3 Concentration–response curves for the five odorants. Error bars represent the SD of the EOG amplitudes for each concentration.

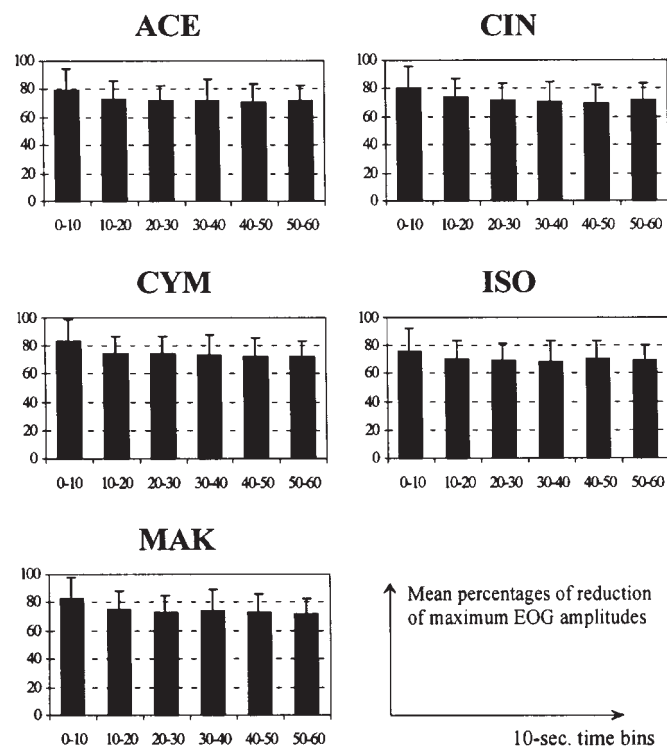


Figure 4 Mean percentage reductions in maximum EOG amplitudes in the six successive 10 s time bins of 60 s odor exposures. They are expressed as proportions of maximum EOG amplitudes in the corresponding odor presentations.

highest decreases were observed for ISO and MAK at both standard and double concentrations.

ACE, CIN and CYM showed simple concentration–response curves over the tested concentration range (Figure 3) whereas the maximum EOG mean amplitudes for ISO and MAK increased in two steps. These amplitudes increased less rapidly than concentration. Depending on the odorants, they were only multiplied by 2.5–5 between the most extreme concentrations.

To analyze the influence of the duration of odor presentation on the EOG signal, odorants were delivered at their standard concentrations over increasing stimulation durations. Figure 4 shows mean EOG amplitudes during six successive 10 s time intervals of 60 s odor exposures. EOG amplitudes did not change significantly over the course of stimulation (Wilcoxon matched pairs signed ranks test, $P > 0.05$), even though a slight decrease was visible for all odorants.

EOG persistence after stimulation

For CIN, ISO, CYM and MAK delivered for 10 s at standard concentrations, EOGs ceased rapidly after the end of the stimulation (upper histogram in Figure 5). In contrast, for ACE, more than 30 extra EOGs, which were greater than the mean spontaneous fluctuations of the

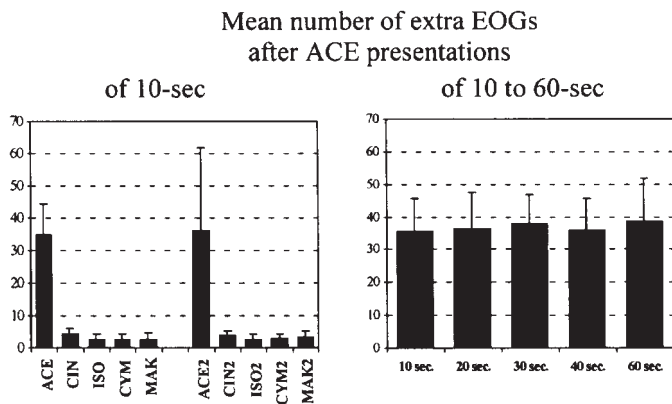


Figure 5 Mean number of extra EOGs after 10 s presentations of the five odorants at their standard and twice standard concentrations (left histogram) and after long duration ACE exposures (right histogram).

signal ± 2 SD were generally evoked after the end of the stimulation period. As shown by the bars ranging from ACE2 to MAK2, this effect was also visible when the concentrations were increased twofold.

Since the persistence of EOGs may depend upon the duration of the stimulation period, increasing durations ranging from 10 to 60 s were tested with ACE and ISO. This did not significantly increase the number of extra EOGs. Between one and four extra EOGs were evoked by ISO (not shown) and between 30 and 40 by ACE (lower histogram in Figure 5), irrespective of the stimulus duration.

Discussion

The present study analyzed EOG responses in the intact nasal cavity of freely breathing animals. First, the data confirm previous observations published in a technical paper (Chaput and Chalansonnet, 1997) that the signal EOG generated by the arrival of odor molecules on the olfactory epithelium is rhythmic and synchronized with respiratory activity. They further show that this signal is greater during the first respiratory cycles following stimulation onset, that it decreases, but remains present, throughout odorant stimulation, even during long odor exposures, and that it may persist for some odors for a relatively long time after the end of stimulation.

Rhythmicity of the EOG signal and its persistence even during long-lasting odor presentations has never before been reported. However, it might be expected in freely breathing animals. Indeed, the arrival of odor molecules is timed by the respiratory flux and the EOG signal resulting from depolarization of the ORNs may be assumed to follow the alternation of the inspiratory phases of sampling of odor molecules and the expiratory phases of odor desorption by the outgoing air. However, this alternation might be modified or even completely suppressed by the anatomy of the nasal pathways, the air velocity in these pathways and/or the physicochemical properties of the

odorants regarding the mucus layer covering the respiratory and olfactory epithelium (see Introduction). Two arguments against such a possibility can be found in the present study: the persistence of EOGs during the whole duration of short (10 s) as well as long duration (60 s) odor presentations and the return of the EOG signal to baseline at the end of each respiratory cycle. This persistence is in agreement with the well-documented respiratory synchronization of olfactory bulb mitral cells, main projection neurons of the ORNs, during short duration stimuli (Macrides and Chorover, 1972; Chaput and Holley, 1980; Chaput, 1986; Chaput *et al.*, 1992; Sobel and Tank, 1993) and long duration (1 h) odor exposures (Chaput and Panhuber, 1982).

EOG amplitudes were relatively low, even at high concentrations, with respect to EOG amplitudes obtained with the same type of electrodes in the olfactory epithelium of tracheotomized rats directly stimulated through an opening performed in the nasal cavity (Duchamp-Viret *et al.*, 1999). Several factors may combine to reduce the number of odorant molecules transported from the entry of the nostrils to the olfactory receptors (Swift and Proctor, 1977; Nachbar and Morton, 1981; Van Drongelen *et al.*, 1982; Getchell *et al.*, 1984; Kurtz and Mozell, 1985; Hornung *et al.*, 1987a,b; Hahn *et al.*, 1993, 1994; Keyhani *et al.*, 1997; Vermeulen *et al.*, 1997). For example, the nasal flow rate and the characteristics of odor molecules, such as their solubility in air and mucus, have been shown to control the access of the stimulus to the receptor cells (Kent *et al.*, 1996). Likewise, the model of Keyhani *et al.* (Keyhani *et al.*, 1997) shows that the adsorption of incoming molecules on the respiratory epithelium likely plays an important role in the processing of olfactory information.

EOG amplitudes were greater in the first cycles of stimulation and then decreased with continued odor presentation. This might represent some form of adaptation to control and limit peripheral olfactory sensitivity (Bray *et al.*, 1998). All OR sites are theoretically unoccupied at the first inhalation and the odorant could potentially occupy all of them (Kamo *et al.*, 1980; van As *et al.*, 1985). After this, the number of unoccupied sites and the total output from the olfactory epithelium should decrease. This might be due to different passive and/or active mechanisms, such as the kinetics of desorption of odor molecules from OR sites or the persistence of odor molecules in their environment.

Maximum EOG amplitudes were increased by ~50% when the concentration was doubled. This sensory compression has already been extensively shown in olfaction and gustation using electrophysiological (Drake *et al.*, 1969; Caprio, 1978, 1982; Derby and Ache, 1984a,b; Johnson *et al.*, 1985) and behavioral (Borroni *et al.*, 1986; Carr and Derby, 1986a,b) concentration-response functions in fishes, decapod crustaceans and amphibians and magnitude estimation functions in human psychophysical studies (Cain, 1975; Bartoshuk and Cleveland, 1977). It is in agreement with Scott *et al.* (Scott *et al.*, 1996), who reported

that most of the concentration–response curves they obtained with their 50 stimuli did not saturate, even with high odor concentrations, that the odorants will be drawn into the nasal cavity by tracheal aspiration or directly applied to the epithelium.

The persistence of numerous extra EOGs after ACE delivery has never before been reported. That surprising observation cannot be explained by odor concentration or by the molecular properties of ACE. Indeed, only a few extra EOGs were observed with much higher concentrations of ISO or MAK (see the upper histogram in Figure 5) and long-lasting ACE exposures did not induce a significantly higher number of extra EOGs (see the lower histogram in Figure 5). Such a phenomenon was not observed with the other ketone (MAK) or the other aromatic hydrocarbon (CYM) included in this study. Further studies will therefore be necessary to analyze this phenomenon.

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