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**Odor response properties of rat olfactory receptor neurons**

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**On the basis of actual studies of molecular biology, a dogma tends to be established: In mammals, odor molecular receptors (ORs) would be highly specific, each one recognizing only one odorant or a small set of structurally similar odor molecules, and a single olfactory receptor neurons (ORN) would express a single OR type. However studies driving the functional expression of ORs fail to demonstrate the low or high specificity of these ORs. Here for the first time in rats, ORNs' selectivity was addressed at the cellular level in physiological conditions using unitary extracellular recordings. Against all expectation, individual ORNs are poorly selective regarding qualitatively distinct odor compounds.**

The chemical receptive field of ORNs mirrors directly the properties of their ORs equipment. This field is defined by the specificity of ORs regarding the structure of odor molecules and by the number and diversity of ORs expressed on the same neuron. Since ORNs were assumed to express only one receptor subtype (1), some authors have postulated that ORNs may have a narrow tuned specificity (2, 3, 4). Furthermore, the expression of ORs has been shown to be spatially segregated and such an organization was proposed as defining the chemotopy of the mucosa (5). Lastly, the rules governing the projection of ORNs to the olfactory bulb seem to maintain the spatial ORNs' segregation (6). Thus, if all these assumptions and results are confirmed, the question of qualitative discrimination of odor molecules would be solved as soon as the ORNs' level through a simple and seducing theory which propose that the olfactory system would function as a "labeled line system" (7).

As reviewed by Duchamp-Viret and Duchamp (8) functional data on the qualitative tuning of ORNs *in vivo* were mainly gathered in amphibians (9) where they fail to support the narrow tuning assumption. Indeed, individual ORNs respond to structurally different odor molecules. This gap between molecular data obtained in mammals and cellular data obtained in amphibians may obviously be ascribed to the phylogenetic evolution, if one assumes that ORNs became more and more selective.

However, such an explanation is not in agreement with the poor selectivity of amphibian (10) and mammalian (11) olfactory bulb mitral cells, the projection-neurons of ORNs, and it may only mask our lack of knowledge of response properties of individual ORNs in intact animals. Thus, the question of the range of the chemical receptive field of individual ORNs was addressed through the use of classical extracellular recording techniques in anaesthetized rats. Regarding the results, the necessity of a re-interpretation of the data of the molecular biology comes in as an open question that will guide the discussion of this paper.

For the first time, individual ORNs were recorded in *in vivo* freely breathing (n=19) or tracheotomized (n=16) rats (12). Ninety ORNs were recorded, generally in the endoturbinates II, during periods ranging from 20 min. to 2 hours. The electro-olfactogram (EOG) was simultaneously recorded as close as possible to the single unit recording site. EOG is a transepithelial potential resulting from the summed activity of numerous ORNs that gives through its kinetic and size a direct and global information on both the intensity of the ORNs' response and the number of responding neurons. Sixteen pure odor compounds were utilized as stimuli. They were selected regarding their effectiveness and their molecular structure from those previously tested in the frog (8). They were chosen as members of the qualitative groups established through several studies by Duchamp and collaborators and belong mainly to the terpene, camphor, aromatic, and straight-chained ketone groups (13). Ethyl vanillin that had never been tested in the frog *in vivo* was added to get information on the IP3 transduction pathway (14). Stimuli were odor pulses of 2-sec. duration delivered at 200ml/min. They were applied directly near the surface of the turbinate using a dynamic multistage olfactometer (15) which ensured a precise control of the concentration range and allowed delivering 12 discrete concentrations. Depending on their saturated vapor pressure (SV), compounds were delivered at concentrations ranging from  $3 \cdot 10^{-8} / 5 \cdot 10^{-7}$  (SV/562) to  $2 \cdot 10^{-5} / 3 \cdot 10^{-4}$  M/l (SV),

In rats, ORNs are spontaneously active. About 40% of them fired spontaneously at more than 100 spikes/min., which is a high rate as compared with rates reported in amphibians (10). Furthermore, they were highly responsive. 83% of neurons were excited by one odor at least. When

considering all stimuli delivered (n=540), 53.5% induced excitatory responses, 5% inhibitory responses and only 41.5% did not evoke a response. Thus, on average, each ORN was excited by 4 odors out of the whole odor set (16). According to the nature of the stimulus, the excitatory power of the odorants varied from 40 to 60%. Only ethyl vanillin was clearly apart, with 15%. ORNs qualitative response spectra were poorly selective. Among the ORNs tested with the whole odor set, many of them were excited by several odors and some of them were even excited by the 16 odorants. The selectivity regarding the odor subset 1 (16) is illustrated in fig. 1. A little more than 25 % of ORNs responded to the six odorants. This is all the more significant as odorants are members of four distinct qualitative groups according to Duchamp and colleagues (7, 10): Camphor for camphor group; limonene for terpene group, anisole and acetophenon for aromatic group and iso-amyl-acetate and methyl-amyl keton for straight-chained keton group. Lastly, whenever a cell responded to none odor of the subset, it never responded to any odor of the whole odor subset 1. Taken together, the present results and previous data gathered in the frog led us to propose the odors of the subset 1 as representative of the qualitative olfactory space and of molecular binding affinities of ORNs.

Twenty-one cells were tested using several concentrations of each odor and their response thresholds were estimated. About fifty percent of ORNs were observed to reach their response thresholds for concentrations higher than SV/10 ( $10^{-6}/10^{-5}$  M/l) while 32% showed supraliminary responses at the lowest available concentration SV/562 ( $10^{-7}/10^{-8}$ ). Some ORNs responded to different odors with thresholds dispersed over a wide concentration range (Fig.2.) while others responded with thresholds that were tuned in a narrow concentration range (fig.3). Increasing concentration was also utilized to gain an insight into the dynamics of ORNs' operation. For most of neurons, the burst response pattern changed progressively, but continuously with concentration: Bursts became more and more sustained and appeared earlier and earlier. Lastly, the responses evoked by the highest concentrations often consisted in an early high frequency and long duration burst or in a decremental initial burst followed by an incremental high frequency and sustained rebound, the delay between the two response phases increasing with concentration. Thus, such an evolution of the response pattern over the whole concentration range, from threshold to high intensities, provides evidence that ORNs did not work at saturation, but on the contrary within a dynamic phase of their excitability.

Simultaneous recordings of the EOG support this assertion since EOG amplitudes increased with concentration, mirroring the recruitment dynamics of ORNs which participate in odor coding.

This study brings for the first time functional evidences that the rat ORNs have broad tuned chemical receptive fields. Their selectivity and sensitivity are fully in agreement with those of mitral cells previously reported in the same animal species (11). By contrast, rats ORNs tend to display a broader qualitative profile and a lower sensibility than those of the frog (17).

As underlined by Zhao and coworkers (4): "Identifying the molecular receptive field of an olfactory receptor neuron is a critical first step in understanding how olfactory perception is achieved by higher brain centers". Here, the chemical receptive fields of ORNs are identified in a biological preparation where they worked as close as possible to physiological conditions.

How can the results of the molecular biology on olfactory receptor proteins be interpreted to take into account our functional results? First off all, it must be noted that the two molecular studies which have addressed the chemical tuning of odor receptors contain some divergent results. Indeed, in insect Sf9 cells transfected with the OR5 receptor, Raming and coworkers (18) have shown that several odor molecules increase IP3 responses and concluded that OR5 are rather poorly selective. By contrast, Zhao and colleagues (4) reported, for the first time in rat ORNs, that increasing the expression of a single gene led to greater responsiveness to octanal and few other compounds with a very close molecular structure. They concluded that they drove the expression of a gene coding for a selective olfactory receptor. It must be noted that the narrow tuned chemical field of their transfected ORNs fits with that of mouse ORNs as previously measured using calcium-imaging (3).

In our recording conditions, the qualitative response profiles of single ORNs directly mirror their ORs' equipment and thus the binding properties of these ORs. If the hypothesis that each ORN expresses one OR is true, the fact that most ORNs respond to several distinct odor molecules demonstrates that ORs are poorly selective. Another possibility is that each ORN would express several ORs of a given subfamily (19), so that its qualitative response spectra would be the sum of the individual receptive fields of its ORs. Lastly, given that some neurons display a differential sensitivity to different odors, we propose that, at the level of a single neuron, not only the categories of ORs and their specificity may differ, but also their number and their affinity for odor molecules.

At a single neuron level, in term of olfactory quality coding, our data are in complete agreement with those previously obtained in the frog. One may argue that in Vertebrates, at the cellular level, the odor/receptor binding process would result in an "across fiber pattern" specification of odor identity and intensity. In other words our results do not support the concept proposed by Axel in a recent congress (7) that there would be "a logic for olfactory perception in which ORNs expressing a given OR, and therefore responsive to a given odor, would project to precise loci in the olfactory bulb". However, the involvement of ORs in a topographical organization of the mucosa and then, in the primary axon targeting in the olfactory bulb is entirely coherent with our results if one ascribes to this topological organization a role in the perpetuation of glomerular innervation despite of the continual renewal of ORNs. Indeed, very recently, the large family of ORs was hypothesized as molecular-addressing code that may serve, not only in the olfactory system but during the ontogenesis, also throughout the entire brain and other organs (20).

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9. In mammals, very few is known on *in situ* ORNs' responses to odorants. To our knowledge, there are only two studies on separated heads of rat embryos and young rats (R.C. Gesteland, C.D. Sigward, *Brain Res.* **133**, 144-149 (1977); R.C. Gesteland, R.A. Yancey, A.I. Farbman, *Neuroscience* **7**, 3127-3136, (1982)) and one study on anesthetized mice (G. Sicard, *Brain Res.* **397**, 405-408, (1986)). In mice, ORNs' extracellular recordings were performed in the posterior septal area using odor stimuli previously used in the frog (G. Sicard, A. Holley, *Brain Res.* **292**, 283-296, (1984)). Mouse ORNs were found to be highly more selective than those of amphibians. When considering all stimuli (n=254), 7.5% evoked excitatory responses against 39% in the frog.
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12. Surgical methods: All experiments were performed following animal care guidelines. Adult Wistar rats (250-300 g) were anesthetized by an intraperitoneal injection of Equithesine (mixture of pentobarbital sodium and chloral hydrate) at the initial dose of 3ml/kg. Anesthetic was then supplemented as necessary to maintain a deep level of anesthesia. Rectal temperature was maintained at  $37 \pm 0.5^{\circ}\text{C}$  by a homeothermic blanket (Harvard Apparatus, USA) and surgical wounds of the animals were regularly infiltrated with 2% Procaine. For recordings, anesthetized animals were secured in a stereotaxic apparatus. Recordings were performed in the Endoturbinat II. Access to the olfactory mucosa was gained by removing the nasal bones and then gently slipping aside the dorsal recess underlying these bones. Recording procedures: Single-unit action potentials were recorded using metal-filled glass micropipettes (3 to 7 M $\Omega$ ), and EOG with a glass micropipettes of 50- $\mu\text{m}$  diameter filled with saline solution. The recorded signals were led through conventional amplifiers. Spike signals were filtered between 300 and 3,000 Hz. Data were stored on the DTR (Data Tape Recorder, by Biologic, France). During the experiment, the single unit nature of the recording was controlled on line by triggering the recorded cell near the background-noise. The activity was monitored on a memory oscilloscope. This allowed us to control the characteristics of the polyphasic spike of the cell studied in order to insure that the same cell was recorded over all the experimental procedure.
13. Studies of ORN qualitative discrimination properties in the frog (8, 10) have shown that the concept of the odor group has a fundamental meaning related to the structure of olfactory molecules.
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16. Since recording a single ORN long enough to test all the 16 odorants was rather difficult, the whole odor-set was subdivided into 3 subsets. Subset 1 comprised camphor (CAM), limonene (LIM), iso-amyl acetate (ISO), acetophenon (ACE), methyl-amyl keton and anisole (MAK). Subset 2 comprised cineol (CIN), vanillin (VAN), p-cymene (CYM), cyclodecanon (CDN), cyclohexanon (HEX) and heptanol (HEP). Subset 3 comprised two pairs of enantiomers: *l*- and *d*- carvone and *l*- and *d*-citronellol. The three subsets were delivered in that order while odors of a given subset were delivered at random. According to this stimulation protocol, only neurons tested with subset 1, at least, were considered to analyze the qualitative discrimination properties of ORNs.
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19. In *in situ* hybridization experiments, Ressler, Sullivan and Buck (*Cell* **73**, 597-609 (1993)) have utilized antisense probes to hybridize ORs subfamilies. They observe a regional distribution of these subfamilies in the olfactory mucosa and hypothesize that each subfamily would code for ORs that would have identical or similar odor specificities. By contrast, our results suggest that a given OR subfamily may code for ORs expressing different odor binding properties. This hypothesis does not call the importance of the regionalization of ORs subfamilies into question since belonging to a subfamily would permit a precise targeting of epithelial zones to the olfactory bulb.
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## Figure captions

Figure 1: Selectivity of ORNs regarding to the odor subset 1 (16). Percentages of recorded ORNs are distributed as function of the number of odors to which they responded by excitatory responses.

Figure 2: Spontaneous activity (upper trace) and representative responses of ORN50 to different stimuli (lower traces). This ORN was tested with all the odors of subsets 1 and 2. All induced excitatory responses. Response to ACE, CAM, CYM are not shown since their thresholds were not estimated. ORN50's thresholds are widely distributed over the available concentration range. For ANI, MAK, ISO, sustained responses were observed for the lowest concentration allowed by the olfactometer (SV/562). Their thresholds were thus lower than  $10^{-7}$  M/l. For XON, the threshold was SV/100 ( $2.5 \cdot 10^{-6}$  M/l). Thresholds were SV/10 for LIM, CIN and VAN, and SV/5 for CDN.

Figure 3: Spontaneous activity (upper trace) and responses (lower traces) of ORN19 to different stimuli delivered at the same concentration in terms of ratio of the saturated vapour pressure of the different compounds (SV/562). Only odors inducing a response are shown. This ORN displayed sustained discharges at the lowest concentration which can be delivered by the olfactometer (from  $2.5 \cdot 10^{-8}$  to  $5.2 \cdot 10^{-7}$  M/l, according to the value of the SV/562 of each compound). Its response threshold was thus overpassed for all these stimuli. This figure indicates further that broad qualitative fields can be observed even at low concentrations, i.e. that one may induce ORNs responses to several different odorants without working at high concentrations.

Figure 4: Spontaneous (upper pair of traces) and odor-evoked (lower pairs of traces) EOGs and single unit responses of ORN55 to increasing concentrations of LIM. This ORN has a low spontaneous firing frequency (about 1.2 Hz on the period shown). The lowest concentration shown ( $1.9 \cdot 10^{-6}$  M/l) induced

a small EOG, but did not modify significantly the ORN firing activity. Increasing the concentration to  $5.9 \cdot 10^{-6}$  M/l (SV/20) induced a larger EOG and a ORN response characterized by a rhythmic discharge composed of 4 spikes. Thus the response threshold of this ORN is comprised between 1.9 and  $5.9 \cdot 10^{-6}$  M/l (i.e. between SV/56.2 and SV/20). Increasing the concentration enhanced the ORN firing activity that became a sustained tonic response pattern, and then an initial high-frequency burst of activity followed by a silence and a rebound. EOG amplitude evolves in parallel : It was very small for  $1.9 \cdot 10^{-6}$  M/l and increased gradually, mirroring the global recruitment of the ORNs situated within the recording field of the electrode. Recordings also show that while the ORN burst discharge frequency increases with concentration, the latency of this discharge shortens with respect to the beginning of the odor-pulse and thus, appears earlier and earlier with respect to the EOG kinetics. Lastly, this figure demonstrates that the concentration range utilized in this study overlaps the dynamic range of rat ORNs' functioning.