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Combined Behavioral and c-Fos Studies Elucidate the Vital Role of Sodium for Odor Detection

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Abstract

Salt, known as taste quality, is generally neglected in olfaction, although the olfactory sensory neurons stretch into the salty nasal mucus covering the olfactory epithelium (OE). Using a psychophysical approach, we directly and functionally demonstrate in the awake rat for a variety of structurally diverse odorants that sodium is a critical factor for olfactory perception and sensitivity, both very important components of mammalian communication and sexual behavior. Bathing the olfactory mucus with an iso-osmotic sodium-free buffer solution results in severe deficits in odorant detection. However, sensitivity returns fully within a few hours, indicating continuous mucus production. In the presence of sodium in the mucus covering the OE, all odorants induce odorant-specific c-Fos expression in the olfactory bulb. Yet, if sodium is absent in the mucus, no c-Fos expression is induced as demonstrated for *n*-octanal. Our noninvasive approach to induce anosmia in mammals here presented—which is fully reversible within hours—opens new possibilities to study the functions of olfactory communication in awake animals.

Key words: c-Fos expression, neural activation, *n*-octanal, odor detection, sodium dependency

Introduction

Olfactory signals are important cues in mammalian life. To determine the biological relevance of odors, it is important to elucidate the mechanisms of olfactory perception. This includes, besides the basic signal transduction cascade, mechanisms of sensitivity and adaptation. Most studies are done on tissue or even cell cultures or in epithelia in the anesthetized animal. However, to really determine the physiological role of odors and analyze the mechanisms involved in odorant perception and coding, it is important to investigate the factors in vivo in the awake freely moving animal. Inhibition of functional activity is a favoured way for olfactory investigations, however it is desirable to produce a transient anosmia that does not harm an animal and is fully reversible. We developed an experimental approach that fulfills these requirements and could unravel the unexpected influence of sodium on odor detection.

In the nasal cavity, volatile chemicals (odorants) bind to seven transmembrane domain receptors (olfactory receptors) (Buck and Axel, 1991) present on the apical cilia of the olfactory receptor neurons (ORNs). In the cilia that stretch into the mucus covering the external surface of the olfactory epithelium (OE), signal transduction commences (Schild and Restrepo, 1998). Binding of an airborne odorant molecule (ligand) to specific G protein–coupled receptors results in the activation of a second messenger cascade, thereby increasing the intracellular cyclic adenosine 3′,5′-monophosphate level, which in turn triggers the opening of cyclic nucleotide-gated cationic channels (CNG channels) (Nakamura and Gold, 1987), calcium influx (Matthews and Reisert, 2003), membrane depolarization, and the generation of action potentials (Kurahashi and Yau, 1993; Shepherd, 1994). Action potentials propagated to specific areas within the olfactory bulb subsequently result in the recognition of odor qualities.

Sensitivity and adaptation to odors depend on several factors in this signal pathway. Electrophysiological recordings from sensory cilia of olfactory receptor neurons *in situ* gave rise to speculations that the ionic composition of the mucus is an important factor for the sensitivity of odor detection (Frings *et al.*, 1991).

However, the technical difficulty of stimulating and recording mammalian olfactory receptor cells at mammalian body temperature has limited most electrophysiological experiments (Reisert and Matthews, 2001). The few studies that

have recorded single-unit discharges from mouse or rat olfactory receptors at body temperature have been carried out in the OE of anesthetized animals (Sicard, 1986; Duchamp-Viret *et al.*, 1999, 2000); others used electroolfactograms (EOGs) in an *in situ* approach (Scott *et al.*, 2000) also in genetically modified animals to investigate signal transduction pathways (Buiakova *et al.*, 1996).

Studies using 2-deoxyglucose autoradiography (Sallaz and Jourdan, 1993; Johnson *et al.*, 2002), visualization of c-Fos mRNA (Guthrie and Gall, 2003), or c-Fos immunohistochemistry (Sallaz and Jourdan, 1993; Klintsova *et al.*, 1995) demonstrate that every odorant activates a unique topography of glomeruli within the main olfactory bulb (MOB). To elucidate neural correlates of our behavioral results, we have used c-Fos expression to provide a global map of neural activity, with single-cell resolution, in the olfactory bulb of *n*-octanal–exposed animals treated with Na^+-containing solution or Na^+ missing in the solution covering the OE.

Materials and methods

Behavioral studies

Odor detection was used as the biologically relevant indicator to investigate the involvement of sodium within the external fluid (mucus) on olfactory sensitivity and signal transduction. Male Wistar rats (age: 200–300 days) were trained in an olfactometer using operant conditioning (Slotnick and Schellinck, 2001) to respond to low concentrations of structurally diverse odorants [ethyl acetate, *n*-hexanal, *n*-octanal, (*R*)-(+)-limonene] but not to clean air. Correct responses (cr) were licking at the reward (water) delivery station only when odor (S+) was presented and not licking when air (S−) was presented. A 100% cr was perfect performance, 50% cr indicated that the animals did not distinguish between odor and air stimuli and responded by chance, and 75% cr level was defined as performance criterion. The cr were rewarded with a predetermined amount of water. In the initial training, we offered an odor vapor at a concentration of 10^{-1} vol%sv (odor concentrations are given as volume percent saturated vapor at about 21°C). As soon as the animals reached a performance level of at least 90% cr, odor concentration was decreased by 0.5 log unit steps until there was a distinct decrement in performance accuracy, and animals failed to reach the performance criterion of at least 75% cr in a block of 40 consecutive trials of a 100-trial session.

Besides, the percentage of cr sampling duration is a reliable indicator for whether an animal is able to detect the odorant (Apfelbach *et al.*, 1990; Slotnick, 1990). Our experimental approach allowed quantification of sample duration on S+ (odor) and S− (air) as a function of odor concentration. At high odor concentrations, sample duration on S+ is short (<1 s) while sampling duration on S− is high. The shorter the sniffing duration, the earlier the rat detects the odor, indicating a high olfactory sensitivity. Prolonged duration of sampling time indicates increasing difficulty or even inability to detect the odorant; the rat "searches" for an odor but does not smell it—possibly as the result of an odor concentration below the threshold level or reduced sensitivity.

After having established individual thresholds for all four odorants, odor concentrations offered in the following experiments were three orders of magnitude above threshold values to ensure that all individuals could detect the respective odorant (Apfelbach *et al.*, 1991).

To modify the composition of the mucus, the olfactory mucosa was washed under slight Halothane anesthesia (Zeneca GmbH, Plankstadt, Germany) with isotonic buffer solutions containing or missing Na^+. Solutions were tested for their osmolarity in a Knauer cryoscopic unit # 24.00 (Knauer, Berlin, Germany). The pH was adjusted to 7.4. To wash the mucus, a catheter was placed into the nostril; 0.5 ml of the respective buffer solution was used for the perfusion of the OE (Kirner *et al.*, 2003). First, we ensured that neither the anesthesia nor the procedure of bathing the mucus by an isotonic/physiological mammalian Ringer's solution applied to the OE affected odor detection performance (Figure 1A). Following established procedures in electrophysiological studies to replace Na^+ in the buffer solution to investigate sodium-dependent currents (Wang *et al.*, 1993; Rabe *et al.*, 1998, 1999), we substituted Na^+ by 2-amino-2-hydroxymethyl-1,3-propandiol (Tris), a large cation with low permeability through olfactory CNG channels (Balasubramanian *et al.*, 1995; Qu *et al.*, 2000) with the solution isoosmolar to Ringer. To ensure that Tris didn't itself have an effect but that the effect was due to sodium, we used Tris-buffer (TB) missing sodium [12.2 g Tris base dissolved in 1 l distilled water; adjusted with HCl to pH 7.4] as well as Tris-buffered saline (TBS) [TB plus 9 g NaCl] or replaced the sodium by choline for isoosmolarity (Hosli *et al.*, 1976; Rink, 1977). In native rat ORNs and recombinant rat olfactory CNG channels expressed in HEK293 cells, the permeability relative to sodium is very low for Tris and even less for choline.

Immunohistochemistry

To support the behavioral data with a second experimental approach, we used the immunohistochemical c-Fos method. Although c-Fos expression in the olfactory bulb is an indirect indicator to show the effect of sodium in odor detection, this method has been shown—at least in some cases—to be even more sensitive than electrophysiological records from olfactory receptor neurons (Lin *et al.*, 2004).

In control rats, we washed the OE with TBS and then exposed the animal for 1 h to clean air. In the first group of experimental animals, the clean air exposure was followed by another 30 min of *n*-octanal exposure. In the second group of experimental animals, the OE was washed with Na^+-free TB solution before the animals were exposed to clean air followed by *n*-octanal. Immediately after the clean air (control group) and odor exposure (experimental groups),

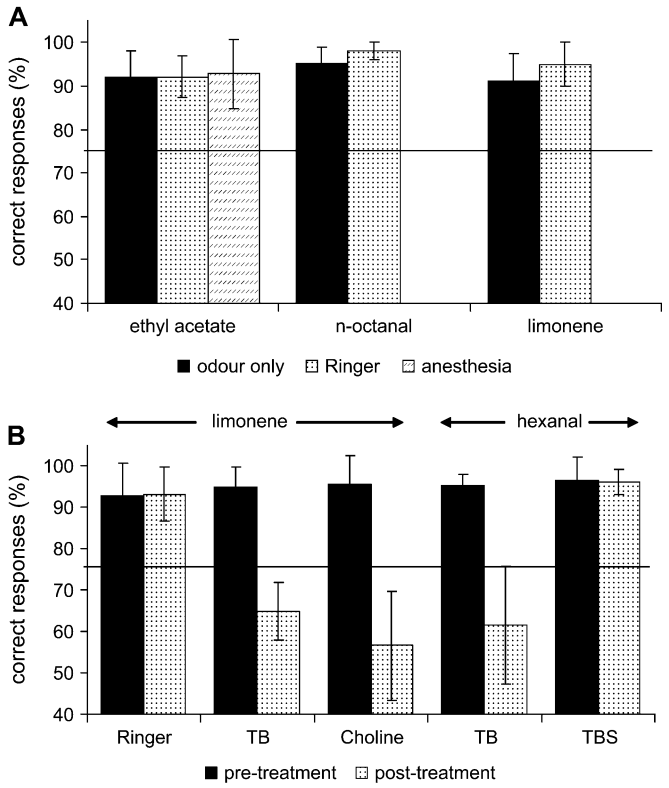

Figure 1 Odor detection performance is **(A)** well above criterion level of 75% cr (indicated by horizontal line) in a 100-trial session tested on a concentration of ethyl acetate (10^{-4} vol%sv), *n*-octanal (10^{-4} vol%sv), and *R*-limonene (10^{-4} vol%sv) prior to treatment. Neither the anesthesia nor the intranasal perfusion with Ringer's solution affected odor detection ability. **(B)** Sodium deficiency of the intranasal perfusion solution resulted in severe deficits in odor detection, shown for the odorants *R*-limonene (10^{-4} vol%sv) and *n*-hexanal (10^{-9} vol%sv). Washing the OE with Na^+-free buffers (TB, choline) reduced olfactory performance below criterion level, whereas Na^+-containing buffers (Ringer, TBS) retained olfactory detection ability, indicating the importance of mucosal sodium for olfactory sensitivity.

animals were sacrificed with an overdose of pentobarbital (intraperitoneally) and perfused intracardially with phosphate-buffered saline followed by 4% paraformaldehyde. For the immunohistochemistry, we applied standard procedures (McGregor *et al.*, 2004). The method described there produces a black oval-shaped immunoprecipitate confined to the cell nucleus of c-Fos positive cells.

From each rat, the right olfactory bulb was cut in coronal sections at 40 μm. Sections were divided in six areas (Figure 3A, A–F) corresponding to the dorsomedial, dorsolateral, medial, lateral, ventromedial, and ventrolateral areas. In every fourth section, c-Fos-immunoreactive cells (only darkly labeled oval-shaped nuclei) were counted microscopically (either a 20× or a 40× objective was used) in the entire glomerular layer of each area by an observer who was blind to group assignment.

Statistical evaluations used the nonparametric Mann–Whitney *U*-test or, where appropriate, the Wilcoxon signed-rank test.

All procedures relating to the care and treatment of rats conformed with the guidelines of the German animal protection laws.

Results

Behavioral results

Bathing the OE with sodium-free TB (pH 7.4 at nasal cavity temperature) reduced the odor detection performance dramatically (<75% cr, Figure 1B). To verify that external Na^+ and not Tris does modify odor detection performance, we applied TBS to the OE; no effect on olfactory sensitivity could be detected: performance levels were as under untreated conditions (96.6 ± 5.8% cr, Figure 1B).

Bathing the OE with a buffer replacing sodium by the impermeant choline resulted in a highly significant decrease of the odor detection ability to chance levels (56.6 ± 13.3% cr, Figure 1B). When replacing the sodium-free solution immediately by a sodium-containing solution, the odor detection performance returned to pretreatment levels (>90% cr). Thus, loss of olfactory performance can be attributed to the missing sodium.

A decreased sensitivity requires higher odorant concentrations for successful odor detection as demonstrated for ethyl acetate. Increase of odor concentration from 10^{-4} to 10^{-2} vol%sv diminished the inhibitory effect of TB on odor detection (95.0 ± 3.5% cr); subsequent lowering of the odor concentration from 10^{-2} to 10^{-4} vol%sv again resulted in close to chance response (65.0 ± 7.0% cr). A recovery effect of olfactory sensitivity within minutes could be excluded by a second increase and decrease in odor concentration that again affected odor detection significantly.

To exclude that odor detection is decreased due to a continuous activation of the receptor cell as a result of increased intracellular calcium, which would cause a continuous spiking of the cell, we measured the odor sampling duration. With decreasing concentrations, sampling duration of S+ increased; near threshold values, sampling duration for S+ equaled or even surpassed that for S−. When Na^+-free solutions were applied to the OE, sampling duration on S+ (mean: 1.3 ± 0.5 s) equaled sampling duration on S− (mean: 1.2 ± 0.6 s): animals were unable to detect the odorant.

Inhibition of odor detection due to missing Na^+ is fully reversible (Figure 2). Within 4 h after treatment with Na^+-free buffer, detection performance returned back to pretreatment levels (ethyl acetate: 90.0 ± 12.2% cr, *n*-octanal: 91.0 ± 10.2% cr). We regard this recovery effect as an indication for the restoration of the external fluid by endogenous mechanisms and gland secretion production and/or the active Na^+/Ca^{2+} pump.

Immunohistochemistry

We undertook an analysis of c-Fos activation by counting stained periglomerular cells in the six areas (Figure 3A,

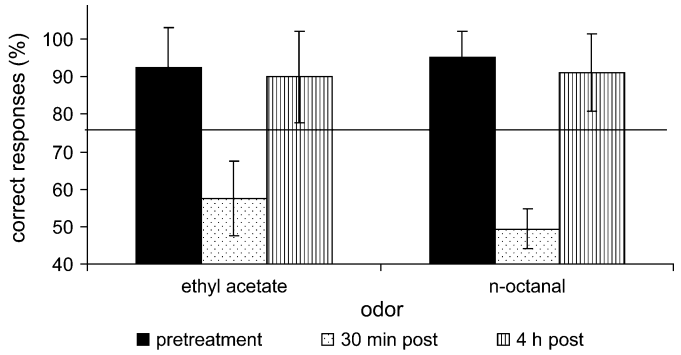

Figure 2 Inhibition of odor detection due to missing Na^+ is reversible. Severe deficits in odor detection were seen for both odorants, ethyl acetate and *n*-octanal, 30 min after washing the OE with Na^+-free buffer. Four hours after treatment with Na^+-free buffer, detection performance returned back to pretreatment levels.

A–F) of the glomerular layer of the MOB. In control animals exposed to clean air only, just few cells expressed c-FOS scattered in various areas of the MOB. The results are shown in Figures 3B, a, and 4A. Since in all cases the number of stained cells were fewer than 10 per area, this finding is considered as random activation.

In *n*-octanal–stimulated animals treated with Na^+-containing buffer solution prior to odor exposure, we found a distinct odor-specific activation pattern. c-Fos-expressing periglomerular cells were not scattered all over the glomerular layer but rather were concentrated around individual glomeruli (Figure 3B, b). This pattern was consistent from animal to animal. High levels of c-Fos activation were seen dorsomedial in areas A, B, and F in the middle of the anterior-posterior extent of the bulb in section numbers 18–27 with the highest peak of activation seen in area B between section numbers 19 and 24 (Figure 4B). In animals treated with Na^+-free buffer solution, the characteristic *n*-octanal activation pattern was not seen (Figures 3B, c, and 4C); the results mimicked those seen in control animals exposed to clean air only. We, therefore, conclude that odor detection was inhibited.

Discussion

In most mammalian species, olfaction is critically involved in behaviors necessary for the survival of the species like food selection, reproduction, defense, and orientation (Eisenberg and Kleinman, 1972). The extent to which olfactory stimuli control behavior of a specific species is thus an important topic of empirical investigation. In many studies, the complete elimination of olfactory sensation is used as a direct experimental approach in the study of olfactory control of behavior. Lesions of the central nervous system as well as manipulations of the OE are assumed to produce anosmia. All these techniques are rather invasive and may severely influence an animal's behavior on several levels.

We assume that with our methodological approach—bathing the OE with sodium-free buffer—fast-diffusing ions are removed from the mucus covering the OE while larger components, such as proteins and polysaccharides, may still be present. It is very unlikely that our approach interferes with odor-binding proteins (OBPs) contained in the mucus. According to some authors (Tegoni *et al.*, 2000), OBPs are active only in the vomeronasal organ since only minor amounts of OBPs are found in the OE. In addition, the physiological role of OBPs remains essentially hypothetical and most probably is not linked to a function of odor transport.

We demonstrated with two different and independent methods, behavior and immunohistochemistry, the inhibitory effect of severely reduced sodium concentrations in the mucus surrounding the olfactory cilia on odor detection and sensitivity. Our approach here presented could well serve to elucidate the role of odorants in awake animals without harming them. Reducing external sodium could affect olfaction in many ways. It seems possible, for example, that it would have direct or indirect effects on the transduction channels and their net currents or that it would block action potentials in the receptor neurons. However, we favor the hypothesis that the reduced external sodium has a severe influence on the Na^+/Ca^{2+} exchanger.

The notion that Na^+/Ca^{2+} exchanger activities may play a major role in extruding calcium ions out of the cell and maintaining Ca^{2+} homeostasis in olfactory receptor cells was assessed by means of laser scanning confocal microscopy in combination with the fluorescent indicators Fluo-3 and Fura-Red (Noe *et al.*, 1997). These data indicate that a high exchanger activity is indeed located in the dendritic knob and probably in the olfactory cilia. Electrophysiological *in vitro* and *in situ* studies support our *in vivo* evidence that the sensitivity is linked to the Ca^{2+}/Na^+ exchange.

Recordings from isolated mouse olfactory receptor cells (Reisert and Matthews, 2001) have allowed a direct comparison of spiking discharges with the receptor currents that gave rise to them in response to well-defined stimuli. These authors demonstrated that the termination of the current mirrors a decline in intracellular Ca^{2+}. This decline is correlated with a decrease in mucosal Na^+ concentrations (Minor *et al.*, 1990, 1992). When sodium was reduced in the external fluid, the second spike (of isolated single cells) of olfactory receptor cells following stimulation was reduced in intensity and increased in duration. This suggests that a Na^+/Ca^{2+} exchange mechanism is involved in the recovery of sensitivity (i.e., adaptation) in individual neurons in frog (Reisert and Matthews, 1998, 1999) and mouse (Reisert and Matthews, 2001). Yet, in these experiments, the neurons were bathed completely in the ionically altered medium; thus, the effect might not be attributed directly or alone to the Ca^{2+}/Na^+ exchanger in the cilia, but somatic effects might have influenced the results on the electrophysiological response. In our experiments, however, only the mucus was altered; thus, we can pin down the sensitivity loss to the mechanisms within

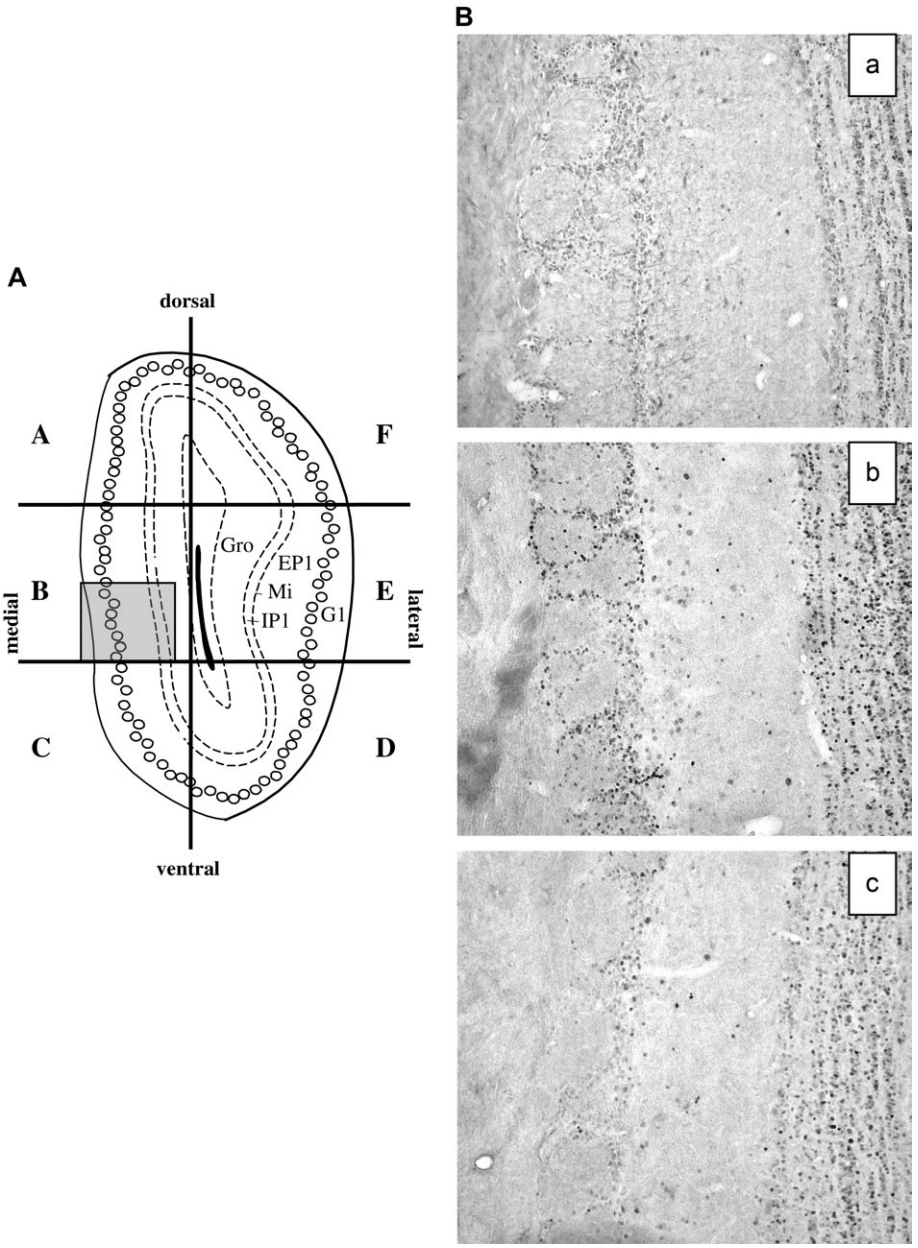

Figure 3 **(A)** Schematic diagram, taken from McGregor *et al.* (2004), showing a coronal section of the MOB indicating the approximate position of the glomeruli (open circles) and indicating the six areas (A–F) in which c-Fos immunoreactivity in the glomerular layer was quantified. **(B)** Photomicrographs (a–c) are taken from the gray square in the medial area B. Photomicrograph (a) represents an air only–exposed control animal; photomicrograph (b) represents an animal exposed to *n*-octanal and treated with Na^+-containing buffer solution, while photomicrograph (c) represents an animal exposed to *n*-octanal and treated with Na^+-free buffer solution. EPL = external plexiform layer; IPL = internal plexiform layer, Gro = granule cell layer, Mi = mitral cell layer.

the signal transduction and adaptation cascade in the cilia. According to present knowledge, a Na^+-dependent Ca^{2+} extrusion mechanism returns cytoplasmatic Ca^{2+} concentration to basal levels after stimulation and mediates the normally rapid recovery of the odor response. Thus, signal transduction is carried by a Ca^{2+} influx, and adaptation occurs if Ca^{2+} is not expelled from the cell. If Na^+ is missing on the outside—which is concurrent with an inactive Na^+/Ca^{2+} exchanger—there is no Ca^{2+} efflux and therefore adaptation. This is consistent with the above-mentioned electrophysiological measurements.

It is interesting to note that the physiological relevance of a change in the olfactory sensitivity due to an alteration in the composition of the mucus is seen, for example, in women during the hormonal cycle (menstrual cycle). Hormones influence the ionic composition of the external mucosal secretion (vaginal mucus), and as a result, the sodium/salt content changes periodically (Macdonald, 1969). Thus, the changes

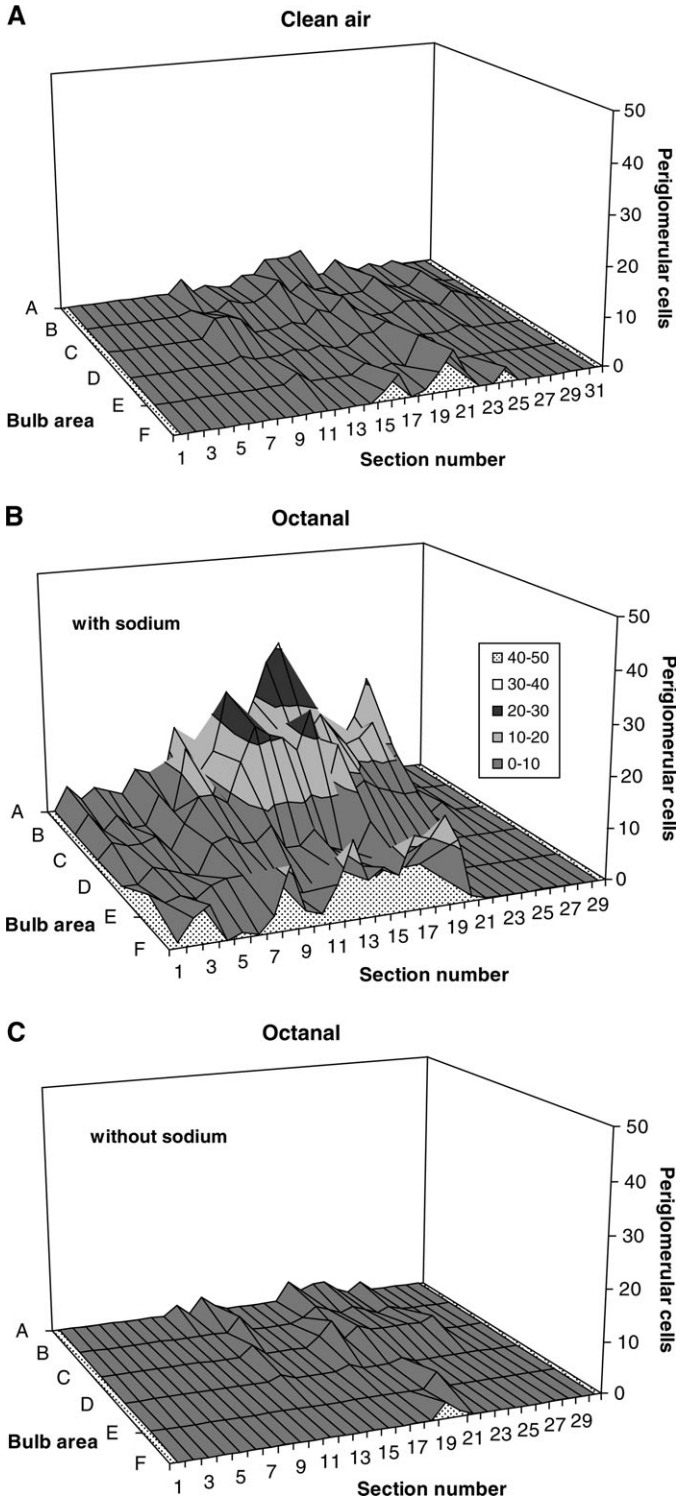

Figure 4 Three-dimensional reconstruction of c-Fos-expressing periglomerular cells in the olfactory bulb. **(A)** Counts in the six areas after 1 h exposure to clean air. **(B)** c-Fos activity pattern after *n*-octanal exposure; before odor stimulation, the OE was washed with buffer solution containing Na^+. **(C)** Neuronal activity pattern after exposure with *n*-octanal; the OE was washed prior to odor exposure with Na^+-free buffer solution.

in salt content are correlated and might be causally linked to olfactory sensitivity changes observed during the menstrual cycle (Achari *et al.*, 1974; Doty *et al.*, 1981). The periodically changing hormone levels can be detected by the hormone receptors located in cells of the Bowman's glands (Stumpf and Sar, 1982) that produce the olfactory mucus and determine its composition. As our study directly demonstrates, salt changes in the mucus modify the sensitivity to odorants. Further, in the OE, sex hormones change the adaptation to EOG responses (Park and Eisthen, 2003). Additionally, sensitivity fluctuations were reported in a number of sensory systems during the menstrual cycle for which the salt content might be responsible, changing membrane potential and the electrophysiological properties of neuronal membranes. With the ability to inhibit odor detection *in vivo*, we can begin to decipher the role of odorants and pheromones in mammalian olfactory communication.

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