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Rats Habituated to Chronic Feeding Restriction Show a Smaller Increase in Olfactory Bulb Reactivity Compared to Newly Fasted Rats

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Abstract

During the 1970s, the multiunit reactivity of the olfactory bulb to food odor was extensively shown to increase before their usual meal in rats habituated to having a single 2 h daily meal compared to the same rats recorded after their usual meal. More recently, we reported dramatic modifications of mitral cell single-unit reactivity in adult rats following a simple a manipulation of the olfactory environment — exposure to an odor. The present study aimed at testing the hypothesis that a simple behavioral change such as habituation to chronic food restriction may induce profound changes in olfactory bulb responsiveness compared to occasional fasting. We compared mitral cell reactivity in non-fasted rats, in rats fasted during 22 h for the very first time, and in rats habituated during 15 days to a chronic 22 h food restriction. Mitral cell single-unit reactivity was found to increase less in rats habituated to fasting than in newly fasted rats. Indeed, the proportion of mitral cell responses to food and non-food odors was significantly higher in rats habituated to fasting than in non-fasted rats, but lower than in newly fasted rats. The proportion of simple unsynchronized and synchronized responses of 1b and 2b types was also lower in habituated rats whereas the proportion of complex synchronized responses of 4b type increased. This decreased responsiveness in habituated rats, similar to that observed in rats repeatedly exposed for 20 min per day to an odor during six consecutive days in our previous studies, is discussed with respect to olfactory bulb plasticity.

Key words: electrophysiological recordings, fasting, food regimen, mitral cell, odor, plasticity

Introduction

Numerous investigations on representational maps in the cortex have recently demonstrated that the functional organization of sensory and motor cortical areas is dynamic and reflects the experiences of the organism [for reviews see (Moore *et al*., 1999; Calford, 2002)]. Somatosensory cortical maps may undergo changes due not only to intensive and long-lasting experience, but also to rapid task-specific behaviors (Birbaumer *et al*., 1997; Karni *et al*., 1998; Moore *et al*., 1999; Braun *et al*., 2000). For example, repetitive motor behaviors or even more simple experimental paradigms such as the repetitive tactile stimulation of a small patch of one digit were found to produce changes in the somatosensory cortex (Jenkins *et al*., 1990; Kleim *et al*., 1998; Braun *et al*., 2001).

In a structure as peripheral as the olfactory bulb (OB), the first relay of the olfactory pathway, important anatomical and functional changes have been observed after a simple manipulation of the olfactory input. Long odor exposures (several months) led to specific patterns of mitral cell degeneration in the OB of adult rats (Døving and Pinching, 1973). Much shorter exposure times (2–8 h) were found to induce important changes in the labeling of olfactory marker protein, a molecular marker specific of olfactory receptor neurons (Yoshihara *et al*., 1993). Lastly a differential expression of the immediate–early genes c-*fos* and c-*jun* was reported in the glomerular and/or granular layers of the OB after odor exposures of short duration (20–30 min) (Sallaz and Jourdan, 1993; Guthrie and Gall, 1995; Baba *et al*., 1997). Functional modifications of the neuronal activity of the OB have also been shown. In rat pups, Wilson and Leon (Wilson and Leon, 1988) have demonstrated that associating an odorant with a positive reinforcement in an associative learning task increases the inhibitory responses of mitral/tufted (M/T) cells, and Skarda and Freeman (Skarda and Freeman, 1987) have reported modifications of the bulbar electroencephalogram map (in the 40–80 Hz band) following an associative learning protocol in adult rabbits.

During the 1970s, OB multiunit reactivity to food odor was extensively show to increase before their 2 h usual meal in rats habituated to have a single daily meal compared to the same rats recorded after their usual meal (Pager *et al*.,

1972; Pager, 1974a,b, 1978; Chaput and Holley, 1976). More recently, we showed for the first time that previous exposure to an odor drastically decreases M/T cell responsiveness in the absence of any experimentally delivered reinforcement (Buonviso *et al*., 1998; Buonviso and Chaput, 2000). This decrease occurred with different exposure odors, but it was not specific of the exposure odor. It concerned only excitatory responses, whether they were characterized by a uniform distribution of activity over the whole duration of the respiratory cycle or by a synchronization of this activity over a phase of the respiratory cycle (Buonviso *et al*., 1992). It persisted in rats re-tested 10 days after exposure and was not reduced by increasing stimulus concentration. In the present study, we tested whether another behavioral modification as simple as a change of food regimen may induce reactivity changes in the OB. We compared M/T cell responsiveness in non-fasted rats, in rats fasted for the very first time during 22 h before recording (newly fasted rats) and in rats habituated during 15 days to a chronic 22 h food restriction (fasting habituated rats), as done by Pager (Pager *et al*., 1972; Pager, 1974a,b, 1978) and Chaput (Chaput and Holley, 1976) during the 1970s. M/T cell reactivity to food and non-food odor stimuli was found to increase less in animals habituated to have a restricted daily food access than in animals fasted for the first time prior to recording.

Materials and methods

Subjects

Experiments were performed on three groups of male Wistar rats (IFFA-Credo) weighing 350–400 g at the time of recordings. They were housed individually at a temperature of 22°C and 50% relative humidity under a 12:12 light–dark cycle (lights on at 20.00 h). They were allowed free access to water. Non-fasted rats (n = 6) were fed *ad libitum*. Newly fasted rats (n = 12) were also fed *ad libitum*, but were deprived of food for 22 h preceding recording. They were recorded at the beginning of the dark period. Food-restricted rats (n = 11) were fed once a day for 15 days before recording. They were allowed free access to food during only 2 h just after the beginning of the dark period. They were recorded just previous to the time of their usual meal, i.e. at the beginning of the dark period, and were thus also fasted for 22 h when recordings were begun.

Electrophysiology

All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the care and use of laboratory animals, and all efforts were made to minimize animal suffering and to reduce the number of animals used. Rats were anesthetized by an i.p. injection of Equithesine (mixture of pentobarbital sodium and chloral hydrate) at the initial dose of 3 ml/kg. Anesthetic was then supplemented as necessary to maintain a deep level of anesthesia, as determined by the depth and rate of the respiratory rhythm of the rat and the absence of leg withdrawal in response to a moderate toe pinch. Temperature was maintained at 37 ± 0.5°C by a homeothermic blanket (Harvard Apparatus, Holliston, MA) and surgical wounds of the animals were regularly infiltrated with 2% Procaine.

Recordings were performed on freely breathing rats. Animals were mounted onto a stereotaxic apparatus and the OBs were surgically exposed. Extracellular unitary activities of M/T cells were recorded with glass micropipettes (10 MΩ) filled with 0.5 M sodium acetate saturated with Chicago Blue Sky (Aldrich). Unitary activities were amplified, band-pass filtered and passed through an amplitude discriminator that sent trigger pulses to a Cambridge Electronic Design (CED) 1401+ system for spike train analysis. The respiratory signal, recorded through a thermistor placed near the entrance of the nostril, was sampled at 200 Hz and sent to the CED system. Recordings were performed all over the ventral and lateral mitral cell layer. Placement of recorded cells in the mitral cell layer was determined during experiments by the occurrence of large-amplitude spikes (Phillips *et al*., 1961). It was verified by marking recording sites at the end of each experiment with small iontophoretic deposits of the Chicago Sky Blue (Aldrich, L'Isle d'Abeau Chesne, France) (Buonviso and Chaput, 1990) and by checking their position in serial frozen section (40 μm thickness) of the OBs stained with Cresyl Violet. Only cells recorded at sites located in the mitral cell layer were retained for the present study.

Experimental protocol began with the recording of 2 min of spontaneous activity. Then stimuli were delivered using a flow dilution olfactometer described in detail elsewhere (Vigouroux and Chaput, 1988). Six odors were used: food odor (FOOD), acetophenon (ACE), cineole (CIN), isoamyl acetate (ISO), *p*-cymen (CYM) and methyl-amyl ketone (MAK). Apart from food odor, obtained from the habitual stock diet of the rat, other odors were delivered at the concentration of 2×10^{-2} of the saturation vapor pressure. Each odor presentation lasted 10 s and was separated from the preceding one by at least 1 min.

Data analysis

The spontaneous activity of the cells and the activity evoked during the presentation of each of the six stimuli were characterized by the mean and maximal firing frequencies during these respective periods, and by their type of discharge pattern along the respiratory cycle, as done in our previous study (Buonviso and Chaput, 2000). Spontaneous mean and maximal firing frequencies were obtained by averaging the corresponding mean and maximal frequencies of twelve 10 s periods. Six periods were taken from the spontaneous activity recorded at the beginning of each cell recording. The other six were taken from the respective spontaneous activity recorded before each odor presentation. The types of discharge pattern were determined with

respect to the spike distribution along the respiratory cycle using histograms triggered by the respiratory cycle and synchronized on the inspiration start. The respiratory cycle was divided into 15 intervals (or bins) of equal duration and histograms were constructed by counting the number of spikes in each bin and averaging it over the different respiratory cycles. The periods used for the histograms for the spontaneous activity and for the odor-evoked activity were, respectively, the 30 s before each stimulation and the 10 s during each stimulation. Discharge patterns were distributed into nine types as described in detail elsewhere (Buonviso *et al.*, 1992). They were classified as 'unsynchronized, simple-synchronized or complex-synchronized' on the basis of the variation of the cell activity along the respiratory cycle (see Figure 2). 'Unsynchronized' patterns (1a, 1b) are characterized by a uniform distribution of the activity along the respiratory cycle; 'simple-synchronized' types (2a, 2b, 3) show a single change in firing activity; and 'complex-synchronized' patterns exhibit multiple activity changes along the respiratory cycle. This classification was done by visual inspection, first independently and then all together by three observers. If at least two of them had chosen the same type for a pattern, they attributed this type to the pattern; otherwise, they decided together the type to which the pattern should be attributed.

Cell responsiveness was determined by comparing each odor-evoked pattern with the corresponding spontaneous pattern obtained from the 30 s period preceding immediately the stimulation. This comparison was done using the probabilistic method recently proposed by Giraudet *et al.* (Giraudet *et al.*, 2002). It consists in calculating how many bins of the two histograms are significantly different at the $P < 0.01$ and $P < 0.001$ levels, assuming that each cycle-triggered histogram is generated by a non-stationary Poisson process, and then determining if at least five bins are significantly different at the 0.01 level or if two bins are significantly different at the 0.001 level.

Results

A total of 44, 61 and 58 cells were recorded, respectively, in non-fasted, newly fasted and fasting habituated rats.

Firing frequencies

Feeding regimen change does not significantly modify mitral cell spontaneous or odor-evoked levels of firing. Indeed, mean and maximal spontaneous firing frequencies were not significantly different in the three rat groups (ANOVA; 0.05 significance level). They were 9.9 ± 6.8 and 111.6 ± 68 spikes per second, respectively, in non-fasted rats, 8.7 ± 5.5 and 75.3 ± 69 spikes per second in newly fasted rats, and 10.5 ± 5.3 and 116 ± 66 spikes per second in rats habituated to fasting.

Odor-evoked mean and maximal frequencies were also not significantly different in the three groups (ANOVA; 0.05 significance level). Odor-evoked mean and maximal firing frequencies were expressed with respect to spontaneous values by calculating the ratio of the corresponding mean and maximal spontaneous firing frequencies of the same cells measured during the 10 s odor presentations and during the preceding 10 s periods of spontaneous activity. A ratio close to 1 indicated an absence of change in frequency from spontaneous to odor-evoked periods. Averaged ratios for mean and maximal frequencies were 1.38 ± 1.1 and 3.96 ± 1.08 in non-fasted rats, 1.59 ± 1.3 and 4.09 ± 1.06 in newly fasted rats and 1.16 ± 0.8 and 3.58 ± 1.15 in fasting habituated rats.

Reactivity

Figure 1 shows the percentage of cells responding to an increasing number of odors and the percentage of cells responding to each odor in the three groups. As visible in the upper diagram and verified by using the median test, there

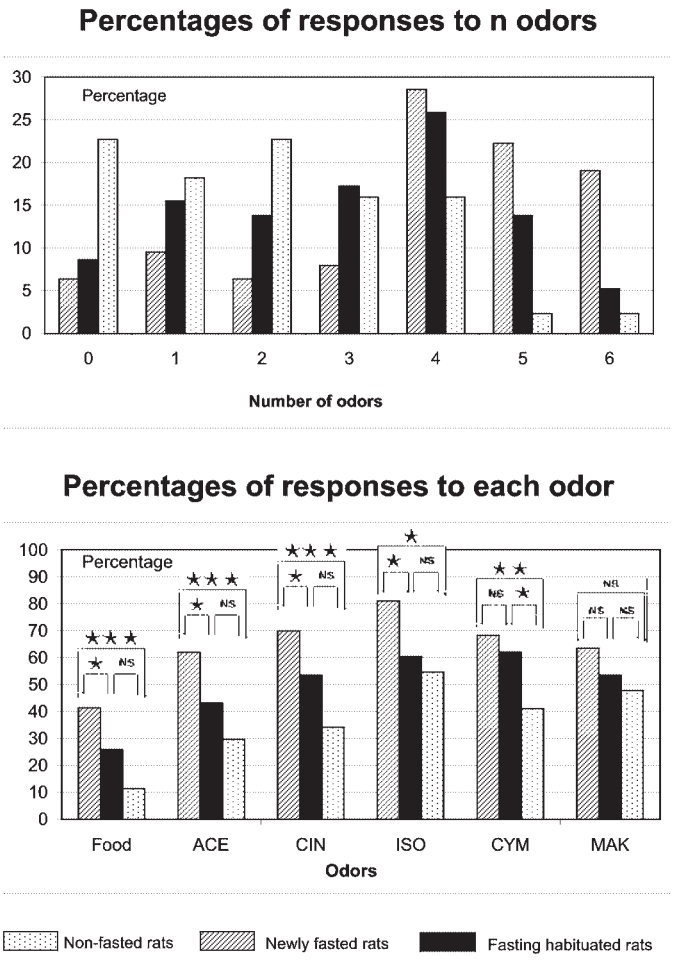

Figure 1 Hatched and black-filled bars present the percentages of cells giving 0, 1, . . ., 6 responses (upper diagram) and the percentages of cells responding to each odor (lower diagram) in newly fasted rats ($n = 61$), in fasting habituated rats ($n = 58$) and in non-fasted rats ($n = 44$), respectively. Asterisks indicate a significant difference, as determined using the chi-square test (*$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$).

was a significant shift from more cells responding up to three odors in the non-fasted group to more cells responding to four or more odors in the fasting habituated group ($0.05 > P > 0.01$). A similar shift occurred from the fasting habituated to the newly fasted group ($P < 0.001$), thus resulting in a highly significant shift from the non-fasted to the newly fasted group ($P < 0.001$).

As shown by the lower diagram, reactivity was significantly lower for all odors in fasting habituated rats than in newly fasted rats and in non-fasted rats with respect to habituated rats (Wilcoxon matched-signed ranks test, $P < 0.05$). Differences between newly fasted and fasting habituated rats reached a statistical significant level for FOOD, ACE, CIN and ISO (chi-square test, 0.05 significance level). Between fasting habituated and non-fasted rats, M/T cell reactivity to CYM only reached a significant level, whereas reactivity to any odor was significantly different between newly fasted and non-fasted rats. Thus, chronic feeding restriction induces a smaller increase of M/T cell reactivity for both food and non-food odor stimuli compared to a first-time feeding restriction.

Temporal patterns

The spontaneous and odor-evoked activities of the cells were classified into types according to their temporal patterns along the respiratory cycle (Figure 2). As shown by the chi-square test, the distributions of spontaneous patterns do not differ significantly in the three rat groups (Figure 2, upper diagram).

Regarding response types (bottom diagram), the percentages of responses of each type for all odors together differ significantly between non-fasted and fasting habituated rats only ($0.01 > P > 0.001$). As shown by individual comparisons done for each type between newly fasted rats and fasting habituated rats, chronic food restriction reduces significantly the probability to observe an unsynchronized response of 1b type or a simple synchronized response of 2b type and increases the probability to observe a complex response of 4b type characterized by a firing decrease followed by a firing increase.

Discussion

The present study shows that a behavioral change as simple as having a single daily meal during 15 days instead of having free access to food causes a drastic and non-specific decrease in M/T cell reactivity. Indeed, as it can be anticipated regarding previous results obtained in our laboratory during the 1970s (Pager *et al*., 1972; Pager, 1974a,b, 1978; Chaput and Holley, 1976), M/T cell reactivity in fasting habituated rats is significantly higher than in non-fasted rats. By contrast, reactivity observed after a 22 h fasting period is significantly lower in fasting habituated rats than in rats fasted for the first time prior to recording.

In earlier studies (Pager *et al*., 1972; Pager, 1974a,b, 1978; Chaput and Holley, 1976), hunger was found to selectively modulate OB responses to food odor and have no significant effect on responses to non-food odor, while non-selective effects were found in this study. However, techniques used in this study were different, and can explain this apparent discrepancy. Indeed, in earlier studies, multiunit activity was recorded in awake animals, while in the present study, single unit activity was recorded in anesthetized animals. Work by Mori *et al*. (Mori *et al*., 1999) and by Ahrens and Freeman (Ahrens and Freeman, 2001) suggests that the activity of local interneurons is necessary for the tuning of mitral cells to specific odors and that, as seen under anesthesia in the present study, single mitral cell selectivity might be disrupted due to a decrease of this local feedback.

The decreased reactivity observed here is as surprising as that we previously found after odor familiarization (Buonviso *et al*., 1998; Buonviso and Chaput, 2000). Indeed, in other sensory systems, changes induced by the habituation or exposure to a particular stimulus are generally specific to that stimulus. For example, habituation was found to produce frequency-selective plasticity in cell receptive fields in auditory cortex (Condon and Weinberger, 1991). Activation produced in the orbitofrontal cortex by the odor of food decreased when food was eaten to satiety, whereas there was no similar decrease for the odor of a food not eaten in the meal (O'Doherty *et al*., 2000). Our results are all the more surprising because a reinforcement of the motivational state of the animals [for a review, see (Toth and Gardiner, 2000)] and thus a selective increase of M/T cell reactivity to food odor were expected. In our exposure experiments, we explained the absence of specificity by the fact that the different exposure odors had different stimulating powers. They activated a different number of receptor cells and therefore a different number of M/T cells. The more M/T cells an odor activate, the more it will overlap with other odors and the more it will affect responses to other odors. This argument cannot explain the general reduction of M/T cell responsiveness observed in this study. Indeed, food odor was not an efficient stimulus for inducing a response. In all rat groups, it was the least efficient stimulus.

The mechanisms underlying the reduction of M/T cell responsiveness observed here after fasting habituation, like the mechanisms involved in the changes in OB responsiveness after odor exposure that we previously reported, are not yet known, even if the control of OB reactivity by higher central nervous structures may be assumed. Numerous centrifugal fibers from different olfactory and non-olfactory cerebral areas have been extensively shown to converge directly or indirectly on the OB (Powell and Cowan, 1963; Powell *et al*., 1965; Price, 1968, 1969; Price and Powell, 1970a,b,c,d), where they terminate on periglomerular and granular interneurons. More recently, the OB has been shown to receive projections from prepro-orexin-positive neurons, which are contained exclusively in the lateral

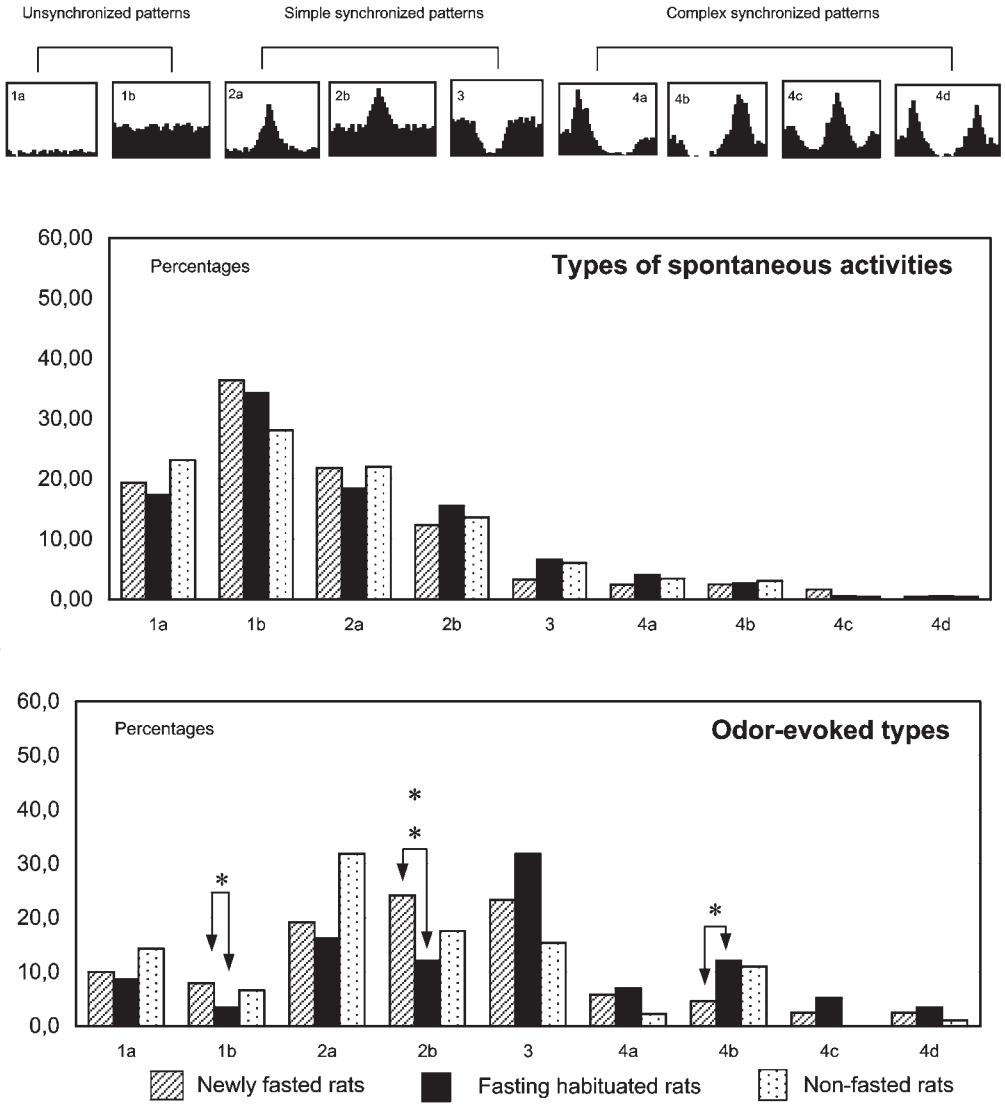

Figure 2 Distribution of the temporal patterns of spontaneous (top bar graph) and odor-evoked (bottom bar graph) activities in newly fasted rats, in fasting habituated rats and in non-fasted rats (hatched, black-filled and dotted bars, respectively). Patterns are schematically represented at the very top of the figure by cycle-triggered histograms in which the *x*-axis represents the duration of the respiratory cycle and the *y*-axis the mean frequency in each time bin. The classification of odor-evoked patterns has been performed only on the activities whose pattern changes from spontaneous to odor-evoked epoch. Asterisks indicate a significant difference, as determined using the chi-square test (*$0.05 > P > 0.01$; **$0.01 > P > 0.001$; ***$P < 0.001$).

hypothalamus, a structure well known for its role in the control of food intake and body weight, and in adjacent hypothalamic areas (Peyron *et al.*, 1998; Nambu *et al.*, 1999). Orexins are neuropeptides involved in the control of feeding and of vigilance, two factors implicated in the modulation of the OB odor reactivity (Pager *et al.*, 1972; Pager, 1974a,b, 1978; Chaput and Holley, 1976, 1980; Gervais and Pager, 1979). Nutritional status regulates the expression of prepro-orexin mRNA (Cai *et al.*, 1999) and orexin levels are higher during wake than during sleep (Kiyashchenko *et al.*, 2002). Administration of orexin has also been shown to promote waking (Piper *et al.*, 2000) and feeding (Dube *et al.*, 1999; Edwards *et al.*, 1999; Sweet *et al.*, 1999). The presence of orexin fibers projecting into the OB along with orexin receptors recently localized in the OB (Caillol *et al.*, 2003) offer the anatomical support for a functional modulation of the olfactory system by orexins. It is therefore possible that the long-term change of food regimen to a single daily meal might influence the OB via the orexinergic system. This modulation of the OB might be brought by orexins either via the centrifugal fibers terminating in the OB or directly via the orexin receptors present in the OB.

In conclusion, the OB seems very sensitive to changes of the internal state of the organism and/or to environmental modifications. If OB responsiveness is so much affected by

the experiences of the organism, the question of the stability of odor coding must be raised, as envisaged in our previous studies. Indeed, origins of changes are relatively trivial in both cases: daily repeated short-duration odor exposures in one case, restricted access to food with <10% body weight loss in the other. In our previous studies, we hypothesized that M/T cells were no more responsive to odors, but that their firing activities in response to the familiar odor were better temporally correlated. A similar effect may be supposed to occur here and further experiments using simultaneous recordings of several M/T cells could be done to explore this possibility.

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