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The olfactory system is affected by steroid aerosol treatment in mice

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Mucignat-Caretta C, Bondí M, Rubini A, Calabrese F, Barbato A. The olfactory system is affected by steroid aerosol treatment in mice. *Am J Physiol Lung Cell Mol Physiol* 297: L1073–L1081, 2009. First published October 2, 2009; doi:10.1152/ajplung.00014.2009.—Asthma needs continuous treatment often for years. In humans, some drugs are administered via aerosol, therefore they come in contact with both respiratory and olfactory mucosa. We explored the possibility that antiasthma corticosteroid treatment could influence the olfactory function by passage through the nose. A group of mice was exposed twice daily for 42 days to fluticasone propionate aerosol and was compared with a control group. Olfactory behavior, respiratory mechanics, histology, and immunoreactivity in the olfactory system were assessed. Fluticasone-treated mice were slower in retrieving a piece of hidden food, but both groups were similarly fast when the food was visible. When a clearly detectable odor was present in the environment, all mice behaved in a similar way. Respiratory mechanics indices were similar in all mice except for the viscose resistance, which was reduced in fluticasone-treated mice. Olfactory mucosa of fluticasone-treated mice was thicker than that of controls. Slight but consistent differences in staining were present for Olfactory Marker Protein but not for other proteins. A mild impairment of olfactory function is present in mice chronically treated with fluticasone aerosol, apparently accompanied by slight modifications of the olfactory receptor cells, and suggests monitoring of olfactory function modifications in long-term steroid users.

corticosteroid; behavior

THE OLFACTORY SYSTEM IS CHARACTERIZED by a rapid turnover of receptor neurons, for which lifespan is ~1 mo, and a neuron turnover in the first relay station, the olfactory bulb (OB). The receptor axons make synapses with dendrites of mitral cells in the glomeruli (11). OB interneurons are continuously renewed by cells that differentiate from neural stem cells residing in the subventricular zone. They arrive into the OB via the rostral migratory stream and are subsequently integrated in OB circuits (25, 33). Other chemosensory organs are present in the nasal cavity, concurring to chemosensory perception (8, 39). This dynamic network is sensitive to environmental challenges, which often result in mucosal damage that is subsequently reflected in OB, affecting olfactory sensitivity (9, 29).

Pathological conditions of the airways may affect the olfactory performance. Both upper and lower airways diseases, for example allergic rhinitis and asthma, are treated with topical administration of corticosteroids. Fluticasone propionate (FP) is one of the most popular and safe treatments for both conditions (2) due to its hepatic metabolism that allows only a topic anti-inflammatory effect without adrenal suppression (31). Whereas the improvement in the pathological state is well-documented (in humans, see, for example, Refs. 13, 24,

30, 34, 37; in mice, Refs. 12, 32), little is known about its possible effect on olfactory cells and in the subsequent olfactory performance.

Corticosteroid inhalatory treatments are often long-lasting, therefore they may influence the quality of life of patients even if they induce only slight side effect. Inhalatory steroid therapy with different drugs gives rise to different olfactory perception, some agents being more pleasant than others (15). Anecdotal reports point to a possible influence of corticosteroid treatment in olfactory sensitivity that can annoy human patients; this issue has never been thoroughly investigated in its biological bases, if any.

To disclose a possible modification of olfactory sensitivity due to FP treatment and differentiate it from the effect on the respiratory pathology, in the present paper, we investigated the effect of FP chronic aerosol treatment on the olfactory performance of mice by analyzing olfactory behavior and histological modifications of the olfactory mucosa and OB. In addition, we investigated the effects of chronic FP treatment on the respiratory mechanics with the end-inflation occlusion method, which allows the description of respiratory mechanics on the basis of a two-compartment model. This method has scarcely been used in mice but has proven to give reliable measurements.

MATERIALS AND METHODS

Animals and treatment. These experiments were approved by the Italian Ministry of Health according to the European law on animal experiments (EEC Directive 86/609/EEC). Twelve adult Swiss female mice were used. Mice were 2 mo old at the beginning of treatment and were housed in two cages hosting six mice each, at $24 \pm 1^\circ\text{C}$, lights on at 6:00 AM for 12 h, with free access to food and water. Every morning, the estrus cycle was monitored via vaginal smear, and animals were weighted and tested for neurological deficits (general condition, deambulation, posture, righting from the side, placing reaction of hindlimbs, geotaxic reaction, avoiding of borders, and equilibrium).

For 42 consecutive days, 6 mice (1 cage) were exposed twice every day to 3 ml of FP (250 μg of FP daily, 41.6 $\mu\text{g}/\text{ml}$, in 9 g/l NaCl, GlaxoSmithKline, Verona, Italy, for a theoretical administration of 20 μg per mouse per day) given via aerosol for 15 min, administered at 1000 and 1800. Mice were put in a closed Plexiglas chamber ($24 \times 13 \times 13 \text{ cm}^3$) with an inlet tube connected to the aerosol apparatus (Clenny Aerosol; Chiesi, Parma, Italy), operated according to the manufacturer's instructions to provide a constant flux to the cage (0.3 ml/min). Control mice ($n = 6$) were exposed for the same time to the same cage without drug administration to balance stress effects between groups while minimizing the risk of cross-contamination due to the repeated air flow in the tubing of the same apparatus. A control experiment was performed on a different set of 12 mice to test the effects of vehicle administration. For 42 days, 6 mice were exposed to vehicle (3 ml of 9 g/l NaCl), and 6 were left in the apparatus for the same time. Under these conditions, no difference was observed in behavioral tests, respiratory mechanics, body weight, histology of the

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lung, and immunohistochemistry on nose and brain tissues. Noteworthy, no difference was observed in the thickness of the olfactory mucosa zone 1, $F(1,10) = 0.6244$, $P = 0.4477$. Therefore, these 2 conditions were considered not different.

Behavioral tests. Tests were run in different days, in a quiet room, starting 35 days after the beginning of aerosol treatment.

The cookie-finding test evaluates olfactory function. Mice deprived of food overnight were put in a cage ($42 \times 25 \times 15 \text{ cm}^2$) in which a food pellet was buried under sawdust and the latency to discover it was measured with a maximum of 5 min. The same test was repeated after 1 h with a food pellet in a visible position in front of the mouse to exclude motor deficits or motivational failure (29).

The dark-light test (28) explores the propensity of mice to exit from a dark compartment to a clear compartment, a behavior modified by the presence of detectable odors in the environment. A plastic cage ($42 \times 25 \times 15 \text{ cm}$; see Fig. S1 available in the data supplement online at the *AJP-Lung Cellular and Molecular Physiology* web site) was divided in a clear compartment and a black one. Five small vials were fixed to the floor. Briefly, the mouse was put in the dark compartment and allowed to move to the other compartment (dark-to-light transition 1). Then, it was removed from the cage and put back in the clear compartment until it moved to the dark side (light-to-dark transition 1). Then, an odor (1:1,000 thymol in paraffin, $10 \mu\text{l}$) was introduced in the vials in the dark compartment, and the sequence was repeated (dark-to-light transition 2 and light-to-dark transition 2). The odor was removed from the dark compartment and then put in the clear compartment before registering dark-to-light transition 3 and light-to-dark transition 3. The cage was washed between the tests with hot water and ethanol to remove traces of odorants. The maximum time for each transition was 3 min. Mice were allowed 1 min rest in their home cage between trials. Data were analyzed with mixed-design ANOVA (group: controls vs. FP-treated; odor: absent, present in the dark, or in the clear compartment; transition: dark-to-light or light-to-dark); post hoc Newman-Keuls tests were run when appropriate.

Statistical analysis. All statistical analyses, as indicated in each section, were performed with Statistica software version 5 '97 edition (<http://www.statsoft.com>). The significance level was set at $P < 0.05$.

Respiratory mechanics. The respiratory mechanics measurements were carried out on five mice in each group; one mouse in each group was discarded for technical problems during measurements. The mean weight for control mice was $35.1 \pm 0.6 \text{ g}$, for FP-treated $34.3 \pm 0.6 \text{ g}$, not different between groups.

The pressure tracings allowed the measurements of respiratory mechanics according to the end-inflation occlusion method (5, 14), as summarized in Fig. 1.

We calculated the total breathing resistance of the lung as $R_{\text{max}} = P_{\text{max}}/Q$ (Q = flow), the Newtonian ohmic resistance as $R_{\text{min}} = P_{\text{min}}/Q$, and the resistance due to pendelluft and stress relaxation as $R_{\text{visc}} = R_{\text{max}} - R_{\text{min}}$. Static lung elastance was also calculated as $E_{\text{st}} = P_{\text{el}}/V_T$ (P_{el} = elastic recoil pressure, V_T = inflation volume).

Four to six inflations were performed consecutively, and the mean values of respiratory mechanical parameters were calculated. Lung hysteresis (Hy) measurements were performed.

Mice were deeply anesthetized ($300 \mu\text{l}$, 20% chloral hydrate in water), and a polyethylene tracheal cannula was inserted into the second tracheal ring and firmly held in place. Mice were positive-pressure ventilated ($V_T = 0.4 \text{ ml}$, breathing frequency = 120/min; rodent ventilator 7025; Ugo Basile, Varese, Italy), the thorax was opened, and the lungs were exposed to atmospheric pressure.

The tracheal cannula was connected to a constant flow pump (SP 2000 Series Syringe Pump sp210iw; World Precision Instruments, Sarasota, FL) set to deliver a V_T of 0.4 ml at a constant flow of 1 ml/s (Q). The time for a complete onset and offset of flow was $\sim 30 \text{ ms}$. Lateral tracheal pressure was monitored (142PC01D; Honeywell, Freeport, IL) and recorded (1326 Econo recorder; Bio-Rad, Milano, Italy). To have a constant volume history, Hy measurements were

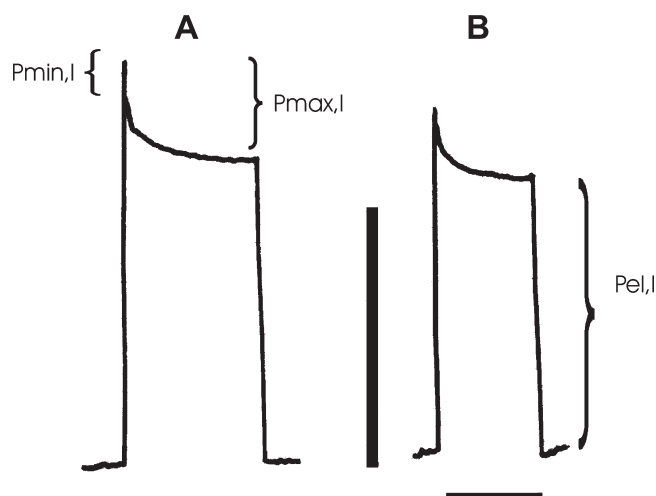


Fig. 1. Examples of respiratory mechanics recordings. Trace A refers to a control mouse, and trace B to a fluticasone propionate (FP)-treated mouse. The vertical thick bar indicates $5 \text{ cmH}_2\text{O}$, and the horizontal thick bar indicates 5 s . The brackets show the extension of the measurement indicated. $P_{\text{min},I}$, ohmic resistive pressure drop; $P_{\text{max},I}$, total resistive pressure drop including viscous pressure dissipation due to pendelluft and stress relaxation; $P_{\text{el},I}$, lung static elastic recoil pressure.

performed after three consecutive lung inflations to a static lung pressure of $20\text{--}25 \text{ cmH}_2\text{O}$.

Equipment resistance for a constant flow of 1 ml/s was separately measured. It amounted to $0.4 \text{ cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$ and was subtracted from results.

Five consecutive stepwise inflations (0.4 ml each) were performed with a precision glass syringe, and the lungs were then deflated in the same manner. The pertinent static P_{el} were measured with a manometer, and the inflation-deflation static pressure-volume curves of the lungs were plotted on a paper of known unit weight. From the weight of the surface encompassed by the static curves, Hy was quantified. The mean values of respiratory mechanical parameters and tissue inflammation indices were calculated and compared (Mann-Whitney test).

Histology and immunohistochemistry. At the end of behavioral tests, mice were returned to their home cage, and FP treatment continued until mice were proven to be in estrus to kill them in a similar hormonal phase, since this can affect mucus and potentially mucosal state. The last morning, mice were given a lethal injection of chloral hydrate, respiratory mechanics measurements were taken (see above), autopsies were performed, and major organs were weighed. The ratio of organ to body weight was analyzed with a monovariate ANOVA for the differences between controls and FP-treated mice; body weight was analyzed comparing both groups at the beginning and at the end of treatment. The OB, lungs, and the nose were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. The noses were decalcified in 5% EDTA, pH 7.4, for 2 wk. Paraffin-embedded sections ($7 \mu\text{m}$) were stained with hematoxylin-eosin (nose and lungs), Heidenhain trichrome (lungs), or Nissl staining (brain), photographed, and measured with Scion Image. The following measurements were taken: the height of the olfactory mucosa in zone 1, calculated as the mean of two measurements taken in the best section from each mouse; and the mean largest diameter of glomeruli in the OB taken along the major axis of the five largest glomeruli in both the lateral and medial part of OB. Glomeruli were chosen from the caudal part of OB since in some animals the glomeruli in the rostral pole were partly damaged. The measurements were taken from the olfactory mucosa of five animals in each group and from the OB of all the six mice in each group and were analyzed with Mann-Whitney U nonparametric test (controls vs. FP-treated mice).

In lung tissue, the severity of inflammatory cell infiltration, the extension of interstitial fibrosis, and the entity of alveolar cuboidalization was evaluated using a semiquantitative analysis (as lung parenchyma involved): score 0, absent; score 1, 1–30%, score 2, >30% <60%; and score 3, >60%.

After preincubation for 1 h with 2% bovine serum albumin and 1% Triton X-100 in PBS, brain sections were incubated at 4°C overnight with one of the following primary antibodies: Olfactory Marker Protein (made in goat, diluted 1:500; OMP; Wako, Neuss, Germany); tyrosine hydroxylase (1:200; Santa Cruz Biotechnology, Heidelberg, Germany); G α protein (made in rabbit, 1:200; Santa Cruz Biotechnology); and synaptophysin (made in mouse, 1:100; Sigma, Milano, Italy). Sections from the nose of each animal were incubated with the OMP antibody only. Fluorescein- or Alexa Fluor-conjugated secondary antibodies (Sigma and Molecular Probes, Milano, Italy, respectively), diluted 1:400, were incubated for 1 h at 37°C.

On nose sections, peroxidase-conjugated secondary antibodies (1:300; Sigma) were also incubated for 2 h and developed with diaminobenzidine tetrahydrochloride. Two independent observers (C. Mucignat-Caretta and M. Bondi) graded the staining with a semiquantitative scoring scale: –, no staining; $\pm$, faint labeling in few cells/fibers; +, faint labeling in most cells/fibers; ++, good labeling in most cells/fibers; +++, strong labeling in all cells/fibers. The nose sections were evaluated for mucosa and nerve staining in zone 1 (dorsal), along the septum up to the border with the respiratory epithelium and in the vomeronasal organ. The OB was evaluated for staining in the medial vs. lateral glomeruli, in the olfactory nerve, and in the accessory OB.

Parallel control slides were prepared either lacking primary antibodies or lacking primary and secondary antibodies or were stained with normal sera to control for background reactivity.

Image management. Photomicrographs from histology or immunohistochemistry slides were acquired with the resident software on a Leica epifluorescence microscope equipped with $\times 20$, $\times 40$, and $\times 100$ objectives, using linear acquisition parameters, no adjustment of tones, and the same acquisition time for both control and treated mice slides. Acquisition times could vary among experiments (each experiment involving a single antibody). Images were acquired at 782×582 pixels in TIFF format. The images were adjusted in brightness and contrast at the same level for control and treated mice, up to a maximum of 5%, using Corel Photo-Paint 12 and then mounted and lettered using Corel Draw 12.

For Fig. 1, original traces were acquired with an Epson scanner at 300 dpi, and then the paper grids were removed with Corel Photo-Paint 12.

RESULTS

Behavioral tests: FP-treated mice are slower in olfactory-driven search. The effects of corticosteroid aerosol treatment on the olfactory performance have never been tested. However, it is possible that corticosteroids act on the olfactory mucosa during passage through the nose. Using behavioral tests, we showed that FP-treated mice were slower in the olfactory-driven search for food. Data from behavioral tests are shown in Fig. 2.

The cookie-finding test showed that aerosol-treated mice were significantly slower in finding a buried food pellet, $F(1,10) = 8.750$, $P < 0.02$, whereas they were as fast as controls if the food was visible, $F(1,10) = 0.030$, $P = 0.865$. This excludes motor deficits or a different drive for food search in FP-treated mice and points to a deficit in olfactory-driven behavior.

The analysis of dark-light test showed a significant effect of group, $F(1,10) = 22.756$, $P < 0.001$, with FP-treated mice

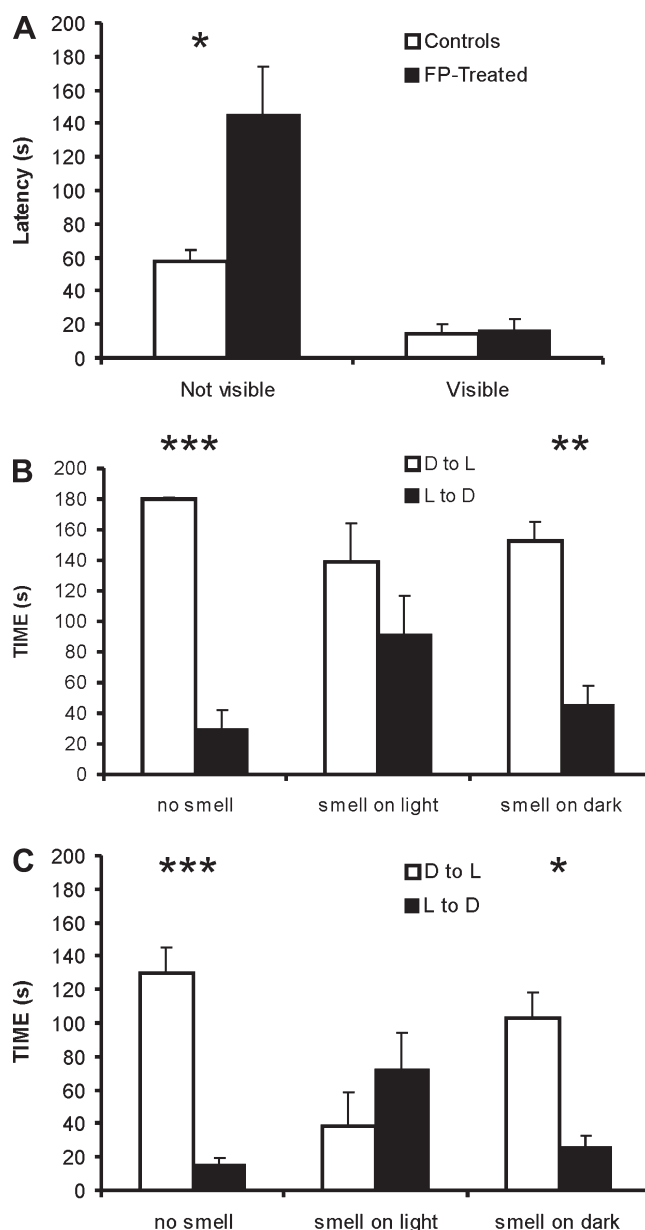


Fig. 2. Data from the behavioral tests. A: cookie-finding test. The mean latency ($\pm$ SE) to recover the food item is shown in white bar for controls and black bars for FP-treated mice. * $P < 0.05$, ANOVA. B and C: dark-light test. The data from control mice are shown in B, and those from FP-treated mice are shown in C. Mean time to cross from 1 compartment to the other: in white bars is the time to cross from dark to light compartment (D to L), in black bars is the time from the clear to the dark compartment (L to D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, ANOVA.

faster than controls (63.9 vs. 106.2 s). Also, the factor transition was significant, $F(1,10) = 53.025$, $P < 0.00005$, with transition from clear to dark compartment faster than the opposite (46.4 vs. 123.7 s). The interaction group $\times$ transition was significant, $F(1,10) = 5.394$, $P < 0.05$. The post hoc test showed that in both groups the light-to-dark transition was faster than the opposite ($P < 0.01$), and that dark-to-light transition was faster in FP-treated compared with control mice ($P < 0.005$), whereas the light-to-dark transition was similar in both groups ($P = 0.26$). The odor $\times$ transition interaction was significant, $F(2,20) = 18.498$, $P < 0.00005$. This interaction

shows that, by collapsing the data of both groups of mice, the light-to-dark transition is faster than dark-to-light ($P < 0.0005$) when the odor is not present (22.1 vs. 154.9 s) or when it is present in the dark compartment (35.4 vs. 127.7 s). On the other hand, when the odor is present in the clear compartment, the dark-to-light and light-to-dark times are similar ($P = 0.65$; 88.6 vs. 81.8 s, respectively); in addition, the dark-to-light time is faster when the odor is in the clear compartment compared with the no-odor and odor-in-dark condition ($P < 0.001$ and $P < 0.02$, respectively). Moreover, the light-to-dark transition is slower when the odor is present in the clear compartment ($P < 0.005$ vs. light-to-dark, no-odor; $P < 0.01$ vs. odor-in-dark).

In summary, the dark-light test indicated the following. 1) Both control and treated mice prefer to stay in the dark compartment and are very fast in crossing from the clear to the dark compartment (no odor condition, light-to-dark transition). 2) When an odor is present in the clear compartment, both control and treated mice promptly exit from the dark compartment to explore the clear, smelly one. When they are put in the clear compartment, they explore it for a longer time, delaying their entry into the dark compartment, overcoming their drive to flight into the dark compartment. 3) When the odor is present in the (preferred) dark compartment, neither control nor FP-treated mice modify their pattern of light-escaping behavior. This may be caused by a ceiling effect of the dark-to-light long transition latencies.

Respiratory mechanics: FP reduces R_{visc} . To dissect the contribution of FP treatment to the respiratory performance from the effect on asthma pathology, the respiratory mechanics parameters were investigated in the same healthy mice.

The mean values of respiratory mechanics parameters are reported in Table 1. The only significant difference was the reduction in R_{visc} of FP-treated mice compared with controls ($P < 0.05$, Mann-Whitney). The other measurements, R_{max} , R_{min} , E_{st} , and Hy, were not statistically different. The reduction in R_{visc} suggested looking for possible morphological alterations in the lung parenchyma.

Histology: FP affects the olfactory mucosa. Given the slight, albeit significant, modifications in the olfactory and respiratory data, autopsies were performed to exclude visible alterations in internal organs, and histological analysis was carried out in the lung, nose, and brain tissues.

Mice did not differ in their body weight at the beginning of the treatment and at death, $P > 0.05$. Also, the weight of different organs (adrenals, lungs, spleen, liver, heart, kidney, and bladder) did not differ between controls and FP-treated mice (Table 2).

Table 1. Respiratory mechanics data

	Control Mice	FP-Treated Mice
R_{max} , cmH ₂ O·ml ⁻¹ ·s ⁻¹	1.98 ± 0.21	1.35 ± 0.23
R_{min} , cmH ₂ O·ml ⁻¹ ·s ⁻¹	0.51 ± 0.12	0.30 ± 0.15
R_{visc} , cmH ₂ O·ml ⁻¹ ·s ⁻¹	1.47 ± 0.10	1.05 ± 0.10*
E_{st} , cmH ₂ O/ml	0.06 ± 0.01	0.07 ± 0.01
Hy, cmH ₂ O/ml	2.03 ± 0.66	1.42 ± 0.53

Data are expressed as means ± SE. E_{st} , static lung elastance; FP, fluticasone propionate; Hy, lung hysteresis; R_{max} , total breathing resistance of the lungs; R_{min} , Newtonian ohmic resistance; R_{visc} , resistance due to pendelluft and stress relaxation. * $P < 0.05$, Mann-Whitney test.

Table 2. Body and organ weight

Organ	Control Mice	FP-Treated Mice
Body before treatment	31.3 ± 0.6	29.4 ± 0.7
Body at death	35.2 ± 0.5	34.5 ± 0.7
Lungs	199.6 ± 8.2	196.1 ± 7.5
Right adrenal	8.1 ± 0.5	7.0 ± 0.6
Left adrenal	6.9 ± 0.6	4.8 ± 0.9
Spleen	167.3 ± 4.2	161.8 ± 12.8
Liver	1,887.7 ± 47.7	2,036.0 ± 117.3
Heart	125.0 ± 2.9	122.8 ± 4.4
Right kidney	239.2 ± 6.2	239.5 ± 11.2
Left kidney	238.7 ± 7.9	234.3 ± 6.5
Bladder	22.3 ± 2.3	20.1 ± 1.4

Data are expressed as means ± SE. Body weight is in grams, organs in milligrams.

Examination of the lung tissue showed that the inflammatory infiltration resulted minimal in both groups and did not change on FP treatment. No significant differences were observed in the amount of inflammation (score 2.8 ± 0.9 in controls vs. 3.0 ± 1.2 in FP-treated mice; $P = 0.901$) and in the extension of fibrosis (score 0 in both groups).

The olfactory mucosa thickness, measured in zone 1 (dorsal), was higher in FP-treated than in control mice ($P < 0.05$, Mann-Whitney): 59.4 ± 1.4 μm for control mice and 65.1 ± 2.1 μm for FP-treated mice.

The lateral glomeruli diameter did not differ between control and FP-treated mice ($P = 0.58$; 103.7 ± 2.1 μm for control mice, 107.3 ± 4.6 μm for FP-treated mice), whereas the diameter of medial glomeruli was larger in FP-treated mice than in controls, $P < 0.05$, 105.9 ± 3.2 μm for control mice vs. 115.1 ± 2.7 μm for FP-treated mice (Fig. 4, C–F).

Therefore, although no morphological modification was detected on the lung tissue, the olfactory mucosa appeared larger after FP treatment. This increase was also reflected on those glomeruli that receive the axons from this area of the olfactory mucosa.

Immunohistochemistry. The increase in the olfactory mucosa thickness could be accompanied by biochemical differences in the cells of the mucosa itself and in the projection areas as well. Immunohistochemistry was performed to investigate the mature olfactory neurons in the mucosa (OMP and G_oα), the synaptic connectivity in the OB (synaptophysin), and the bulbar dopaminergic interneurons (tyrosine hydroxylase). In this way, we could explore the downstream effectors of the olfactory receptor cells at the first central station.

Representative sections are shown in Figs. 3–6. In the nose, OMP labeling was evident along the whole neuroepithelium of both control and FP-treated mice. It was very strong in the olfactory fila, whereas in zone 1 (dorsal) of the epithelium it was slightly more intense in the FP-treated mice. On the other hand, the vomeronasal neurons appeared intensely stained in both groups (Fig. 3, A–D, and Fig. 4, A and B).

In the OB, OMP labeling was evident in the glomeruli and in the nerve layer (Fig. 3, E–H). Control mice showed a more intense labeling compared with FP-treated. Their lateral glomeruli appeared more intensely labeled than the medial, a difference not apparent in FP-treated mice. Also, glomeruli in the accessory OB were intensely labeled in control mice, as in FP-treated (Fig. 5, A and B). No difference in G_oα staining was apparent between the main OB of both groups, since in both

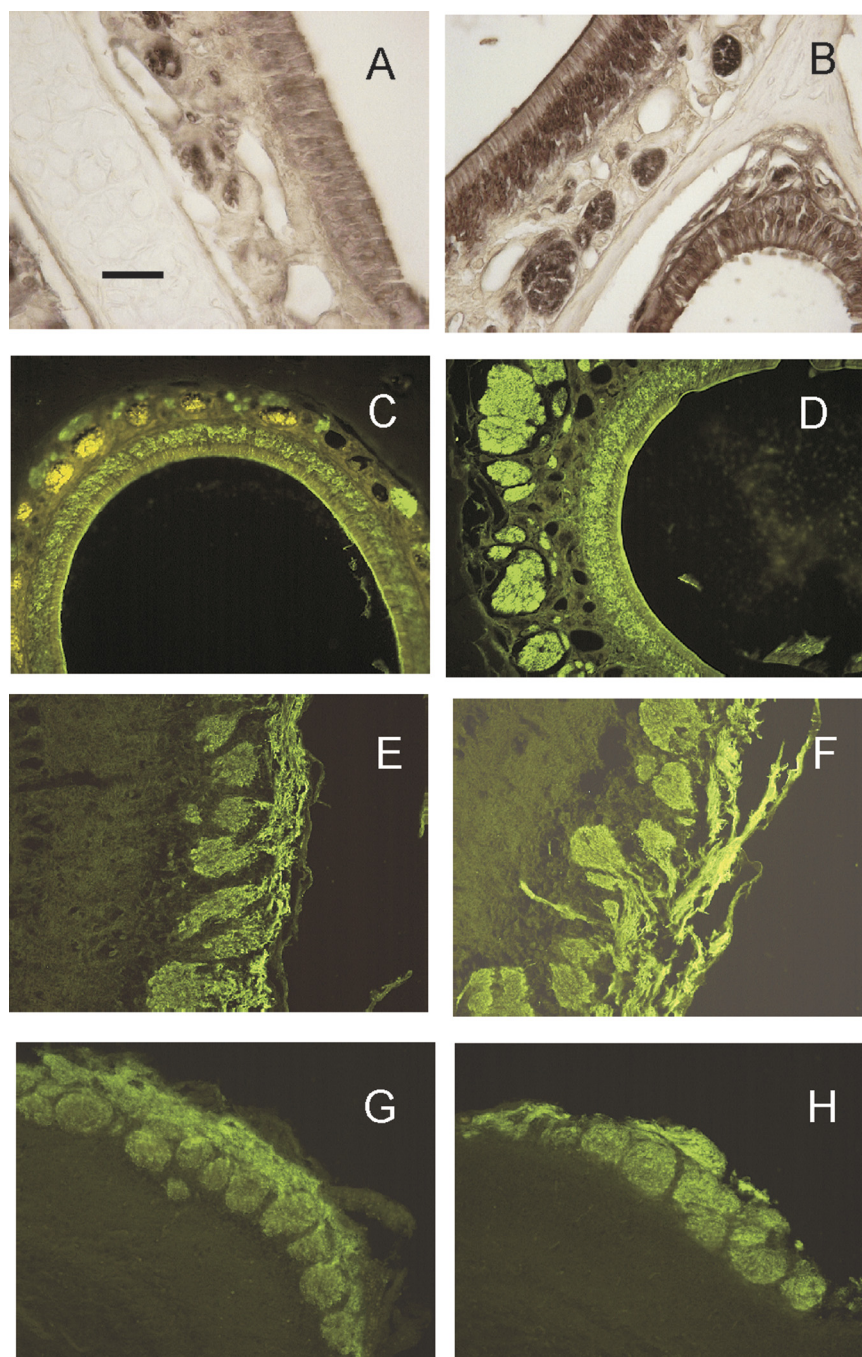


Fig. 3. Immunohistochemistry of representative sections of olfactory mucosa and olfactory bulb. *A*: Olfactory Marker Protein (OMP) immunolabeling of the olfactory mucosa in a control mouse. The olfactory receptor neurons and the olfactory fila are labeled. *B*: OMP immunolabeling of the olfactory mucosa in a FP-treated mouse. *C*: OMP immunolabeling of the olfactory mucosa in a control mouse, dorsal on the top. Some autofluorescence is evident in yellow. The whole zone 1 is shown in both *C* and *D*. *D*: OMP immunolabeling of the olfactory mucosa in a FP-treated mouse, dorsal on the left. The intensity of staining is apparently higher than in *C*. *E–H*: OMP immunostaining of the olfactory bulb glomeruli. *E* and *F*: control mouse. *G* and *H*: FP-treated mouse. *E* and *G*: medial glomeruli. *F* and *H*: lateral glomeruli. The control mouse shows a more intense labeling in the lateral glomeruli. The labeling in FP-treated mouse is fainter than in the control mouse. Bar: 25 μ m for *A* and *B*, 50 μ m for *C–H*.

groups the lateral glomeruli were labeled better than the medial (Fig. 5, *C–F*). A similar pattern was also obtained with synaptophysin, with a stronger labeling of lateral glomeruli in both groups (Fig. 6, *A–D*). A different result was obtained with tyrosine hydroxylase that strongly stained dopaminergic periglomerular cells and fibers within the glomeruli. The labeling was stronger in medial glomeruli, compared with lateral ones, in both control and FP-treated mice (Fig. 6, *E–H*).

DISCUSSION

The olfactory system is directly exposed to the airstream, making it vulnerable to airborne substances. Prolonged treatments for pathological conditions often affect the quality of life

of patients, therefore the safety of drugs for nonvital systems, like the sense of smell, should be considered. In this study, we explored the possibility that a drug commonly used for the treatment of asthma and allergic rhinitis could affect olfaction in normal mice by acting on the olfactory mucosa en route to the lungs.

In patients, aerosol FP may be administered once or twice daily for long periods, therefore we cannot exclude an effect on the olfactory mucosa as well. FP is a lipophilic drug that preferentially partition in the systemic tissue compartment and thus has a large volume of distribution at steady state that acts as a slow release reservoir (24). On the other hand, the high lipophilicity diminishes the solubility in water, and hence in

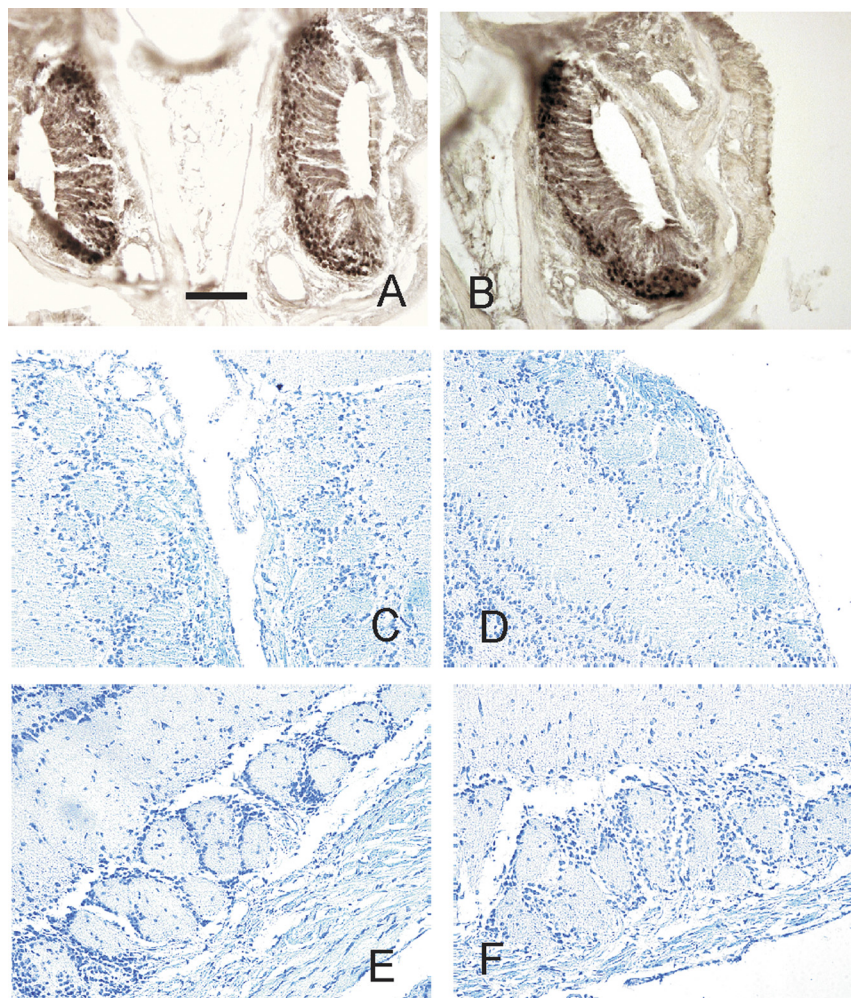


Fig. 4. A: OMP immunolabeling of the vomeronasal organ of a control mouse. B: OMP immunolabeling of the vomeronasal organ of a FP-treated mouse. C–F: Nissl-stained horizontal olfactory bulb sections. C and D: control mouse, medial and lateral glomeruli, respectively. E and F: FP-treated mouse, medial and lateral glomeruli, respectively. Bar: 50 μm .

mucus, presumably enhancing the quantity of drug cleared away by ciliary movement. However, in the human nasal mucosa, FP was shown to persist for several hours, therefore it can exert its effect for a prolonged time (7): albeit mice have a faster metabolism, we mimicked the human treatment by exposing mice twice daily to FP. The use of topic FP induces similar changes in inflammatory responses in both the upper and lower airways (16) by reducing the number of eosinophils and activated lymphocytes (20, 23) and consequently inhibits the local mediator activity (27): the histology data confirmed that the inflammatory infiltration of the lung tissue resulted minimal in both groups, and no significant difference was found as an effect of FP treatment.

The mean values of $R_{\min}$, $R_{\max}$, R_{visc} , and E_{st} reported in the literature for healthy mice range between 0.33 and 1.36 $\text{cmH}_2\text{O}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$, 0.95 and 2.09 $\text{cmH}_2\text{O}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$, 0.62 and 0.8 $\text{cmH}_2\text{O}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$, and 17.8 and 38.5 $\text{cmH}_2\text{O}/\text{ml}$, respectively (10, 35, 40). The present results are within the same range. We found somewhat lower values of E_{st} , but the mean body weight of our mice was higher than those of mice used by others authors.

Hy values were measured for a V_T of 2 ml and were also similar to those previously reported (36, 38), which range from 2 to 3 $\text{cmH}_2\text{O}/\text{ml}$, however, for smaller V_T . We hence confirm that healthy mice lungs exhibit hysteretic behavior.

At present, a positive effect of FP aerosol has been shown on bronchial responsiveness and airway inflammation in bronchitic cats (21) and mice (12). Positive effects were also reported for human asthmatic subjects (13, 34, 37) and chronic obstructive pulmonary disease patients (30), but no data are available describing possible effects of FP on normal lungs.

The only significant effect of FP was a reduction of R_{visc} . Apparently, FP aerosol in healthy mice significantly reduces the resistive work of breathing, which is mainly due to stress relaxation and pendelluft (5, 14).

We suggest that FP may change the mechanical characteristics of the alveolar wall and associated collagen fibers, thus modifying the viscoelastic properties of the lungs and stress relaxation. The dishomogeneity of peripheral airways resistance might be reduced as well, leading to a decrease of pendelluft and R_{visc} .

Similar to previous data in humans (30), we did not find E_{st} changes, suggesting lung tissue stiffness is not influenced by FP aerosol.

We conclude that FP aerosol reduces airways resistance, at least in its viscoelastic component and also in healthy mice, in the absence of significant airways or lung tissue inflammation.

No reports are available on the effect of FP on the olfactory function except for the initial but persistent improvement in olfaction in hyposmic patients due probably to the reduction in

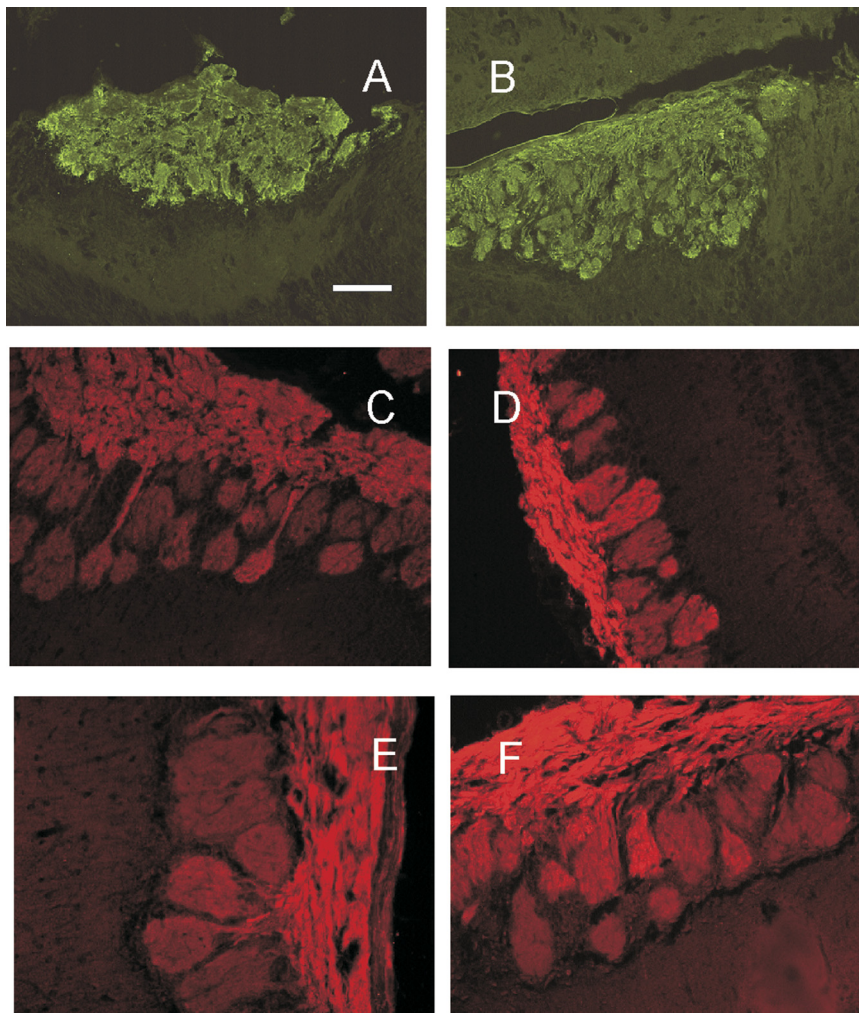


Fig. 5. Immunolabeling of the olfactory bulb. Caudal on the top for A and B. A: OMP labeling in the accessory olfactory bulb of a control mouse. The glomeruli are heavily stained. B: OMP labeling in the accessory olfactory bulb of a FP-treated mouse. The glomeruli are heavily stained, similar to control. C–F: $G_{\alpha\alpha}$ immunoreactivity of the olfactory bulb. C and D: control mouse, medial and lateral glomeruli, respectively. E and F: FP-treated mouse, medial and lateral glomeruli, respectively. In both control and FP-treated mouse, the immunolabeling of lateral glomeruli and olfactory nerve appear more intense. Bar: 50 μ m.

inflammatory processes (6). This result apparently contrasts with the present behavioral findings, since FP-treated mice were impaired in their ability to use olfactory information to reach an invisible food item. However, our mice were healthy, therefore the effect on olfactory behavior, together with the changes in olfactory mucosa thickness and OMP reactivity, can suggest an effect of FP on mucosa cytology that, in turn, acts on olfactory function.

Regular use of FP spray apparently does not damage the olfactory mucosa (22). On the contrary, after 1 yr of FP treatment, the human nasal epithelium thickness increases, an effect not observed with anti-inflammatory nonsteroid drug treatment: this increase in thickness was interpreted as a repair process from epithelial damage, induced by chronic allergic inflammation (4). This result parallels our finding in mice nose, in which we also observed an increase in mucosa thickness but in the absence of previous inflammatory processes. A possible explanation, different from the mucosal protective mechanism, refers to the effects of glucocorticoid treatment that may affect the water and sodium transport across the epithelium, through the ENaC channels (3). The increase in mucosa thickness is conceivably related to the larger dimension of glomeruli in the zone 1 projection area, that is, in the medial glomeruli, compared with the lateral glomeruli, which appeared unchanged. The increased thickness of olfactory mucosa appeared accom-

panied by an increase in labeling with OMP, a mature olfactory neuron marker. This would suggest a higher number of olfactory receptor cells, and hence of axons reaching the glomeruli, accounting for their larger diameter. It is known that severing the olfactory axons, or making the olfactory receptor neurons die, reduces the diameter of glomeruli in OB so that the glomeruli dimension reflects the number of axons therein (18). OMP is a marker for mature olfactory neurons, and its expression reliably changes according to the functional state of the cell. The decrease in glomerular labeling in FP-treated mice, contrary to the increase in mucosa labeling, possibly reflects the different structure or the newer formation of these larger glomeruli: the fluorescence signal appears fainter if diluted over a larger area, as it happens in the glomeruli, contrary to what happens in the mucosa. All the other markers that we have used, $G_{\alpha\alpha}$ as an olfactory neuron marker, synaptophysin as a central synapses marker, and tyrosine hydroxylase as a dopaminergic marker, appeared unchanged following FP treatment; this suggests that FP can act locally in the mucosa to alter the receptor lifespan/turnover. This, in turn, affects the first synapse in the olfactory glomeruli and can result in the impairment of the olfactory-guided behavior that was detected. It is known that in the OB the glutamatergic mitral cells modulate GABAergic synapse by releasing metabolites of deoxycorticosterone (1). It remains unclear whether this pos-

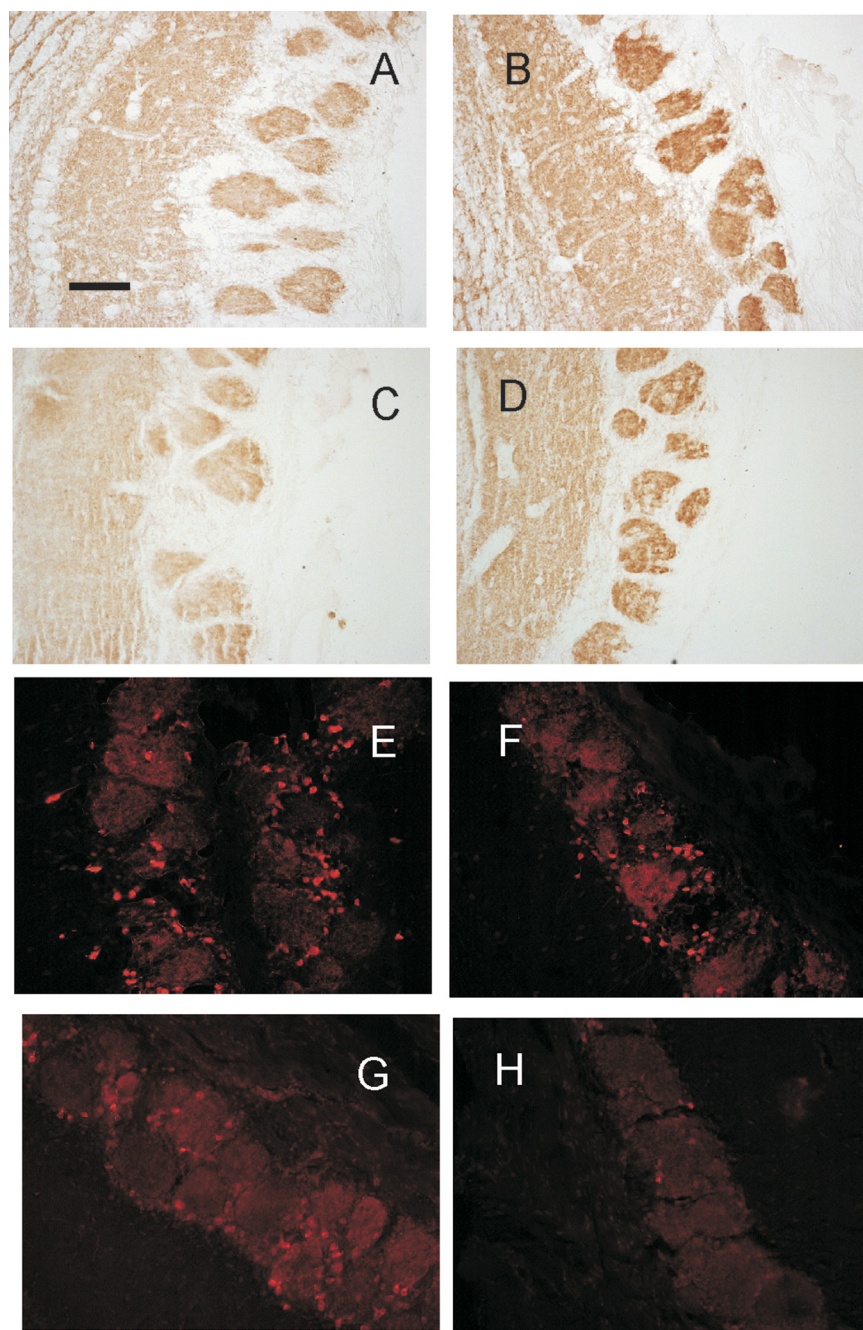


Fig. 6. Immunolabeling of the olfactory bulb. *A–D*: anti-synaptophysin labeling. *A* and *B*: control mouse, medial and lateral glomeruli, respectively. *C* and *D*: FP-treated mouse, medial and lateral glomeruli, respectively. *E–H*: tyrosine hydroxylase immunoreactivity. *E* and *F*: control mouse, medial and lateral glomeruli, respectively. *G* and *H*: FP-treated mouse, medial and lateral glomeruli, respectively. For synaptophysin, the labeling is more evident in lateral glomeruli, whereas medial glomeruli are more intensely labeled with tyrosine hydroxylase. Bar: 50 μ m.

sibility also exists for the glutamatergic olfactory sensory neurons; our data apparently support an action of the corticosteroid FP on olfactory sensory neurons.

Other gross modifications of the OB circuits were not apparent using the synaptophysin and tyrosine hydroxylase markers. This can help in confining the deficits to the first step of olfactory detection, as it is apparent from the second behavioral test, in which the odor was clearly above threshold, and mice behave in a similar way in both groups.

Most data on allergic rhinitis and asthma management are obtained directly from human patients examination. The function of mucosa is greatly affected by the composition of the mucus layer, which is regulated by controlling sodium and chloride ions. In normal human subjects, the treatment with FP

apparently potentiates mucin secretion while not changing serous secretion or microvascular permeability (26). On the other hand, FP increases sodium absorption as measured by changes in the voltage across the epithelium (17) and slows the ciliary beat frequency (19). It is possible that these effects on mucus and ciliary movements would affect olfaction as well.

In conclusion, these data show that chronic aerosol FP treatment can affect the olfactory function in mice and suggest to monitor olfactory function modifications in patients.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES

- Agis-Balboa RC, Pinna G, Zhubi A, Maloku E, Veldic M, Costa E, Guidotti A. Characterization of brain neurons that express enzymes mediating neurosteroid biosynthesis. *Proc Natl Acad Sci USA* 103: 14602–14607, 2006.
- Bachert C, El-Akkad T. Patient preferences and sensory comparisons of three intranasal corticosteroids for the treatment of allergic rhinitis. *Ann Allergy Asthma Immunol* 89: 292–297, 2002.
- Bangel N, Dahlhoff C, Sobczak K, Weber WM, Kusche-Vihrog K. Upregulated expression of EnaC in human CF nasal epithelium. *J Cyst Fibros* 7: 197–205, 2008.
- Baroody FM, Cheng CC, Moylan B, deTineo M, Haney L, Reed KD, Cook CK, Westlund RE, Sengupta E, Corey JP, Togias A, Naclerio RM. Absence of nasal mucosal atrophy with fluticasone aqueous nasal spray. *Arch Otolaryngol Head Neck Surg* 127: 193–199, 2001.
- Bates JH, Rossi A, Milic-Emili J. Analysis of the behavior of the respiratory system with constant inspiratory flow. *J Appl Physiol* 58: 1840–1848, 1985.
- Blomqvist EH, Lundblad L, Bergstedt H, Stjarne P. Placebo-controlled, randomized, double-blind study evaluating the efficacy of fluticasone propionate nasal spray for the treatment of patients with hyposmia/anosmia. *Acta Otolaryngol (Stockh)* 123: 862–868, 2003.
- Bonsmann U, Bachert C, Delank KW, Rohdewald P. Presence of fluticasone propionate on human nasal mucosal surface and in human nasal tissue over a period of 24 h after intranasal application. *Allergy* 56: 532–535, 2001.
- Breer H, Fleischer J, Strotmann J. The sense of smell: multiple olfactory subsystems. *Cell Mol Life Sci* 63: 1465–1475, 2006.
- Calderon-Garciduenas L, Franco-Lira M, Torres-Jardon R, Henriquez-Roldán C, Barragán-Mejía G, Valencia-Salazar G, González-Maciel A, Reynoso-Robles R, Villarreal-Calderón R, Reed W. Pediatric respiratory and systemic effects of chronic pollution exposure: nose, lung, heart, and brain pathology. *Toxicol Pathol* 35: 154–162, 2007.
- Carvalho RS, de Pinho AG, de Andrade AP, Silva CA, Gaio CE, Tavares P. Analysis of pulmonary mechanics in an experimental model of sepsis. *J Bras Pneumol* 32: 316–321, 2006.
- Chen WR, Shepherd GM. The olfactory glomerulus: a cortical module with specific functions. *J Neurocytol* 34: 353–360, 2005.
- Chu HW, Campbell JA, Rino JG, Harbeck RJ, Martin RJ. Inhaled fluticasone propionate reduces concentration of Mycoplasma pneumoniae, inflammation, and bronchial hyperresponsiveness in lungs of mice. *J Infect Dis* 189: 1119–1127, 2004.
- Corsico A, Pellegrino R, Zoia MC, Barbano L, Brusasco V, Cerveri I. Effects of inhaled steroids on methacholine-induced bronchoconstriction and gas-trapping in mild asthma. *Eur Respir J* 15: 687–692, 2000.
- D'Angelo E, Calderini E, Torri G, Robatto M, Bono D, Milic-Emili J. Respiratory mechanics in anesthetized paralyzed humans: effects of flow, volume, and time. *J Appl Physiol* 67: 2556–2564, 1989.
- Doty RL, Bromley SM. Effects of drugs on olfaction and taste. *Otolaryngol Clin North Am* 37: 1229–1254, 2004.
- Durham SR. Mechanisms of mucosal inflammation in the nose and lungs. *Clin Exp Allergy* 28: 11–16, 1998.
- Graham SM, Scott SN, Launspach J, Zabner J. The effects of fluticasone propionate on nasal epithelial potential difference. *Am J Rhinol* 16: 145–149, 2002.
- Graziadei PP, Monti-Graziadei GA. Neurogenesis and neuron regeneration in the olfactory system of mammals. III. Deafferentation and reinnervation of the olfactory bulb following section of the fila olfactoria in rat. *J Neurocytol* 9: 145–162, 1980.
- Hofmann T, Gugatschga M, Koidl B, Wolf G. Influence of preservatives and topical steroids on ciliary beat frequency in vitro. *Arch Otolaryngol Head Neck Surg* 130: 440–445, 2004.
- Holm AF, Godthelp T, Fokkens WJ, Severijnen EA, Mulder PG, Vroom TM, Rijntjes E. Long-term effects of corticosteroid nasal spray on nasal inflammatory cells in patients with perennial allergic rhinitis. *Clin Exp Allergy* 29: 1356–1366, 1999.
- Kirschvink N, Leemans J, Delvaux F, Snaps F, Jaspert S, Evrard B, Delattre L, Cambier C, Clercx C, Gustin P. Inhaled fluticasone reduces bronchial responsiveness and airway inflammation in cats with mild chronic bronchitis. *J Feline Med Surg* 8: 45–54, 2006.
- Klimek L, Bachert C, Hormann K. Steroid sprays in non-infectious rhinitis and sinusitis. Proper and regular spraying does not damage the nasal mucosa. *MMW Fortschr Med* 144: 41–43, 2002.
- Levenson T, Greenberger PA. Immunologic effects of intranasal corticosteroids. *Allergy Asthma Proc* 17: 157–159, 1996.
- Lipworth BJ, Jackson CM. Safety of inhaled and intranasal corticosteroids: lessons for the new millennium. *Drug Saf* 23: 11–33, 2000.
- Lledo PM, Lazarini F. Neuronal replacement in microcircuits of the adult olfactory system. *C R Biol* 330: 510–520, 2007.
- MacGregor FB, Robson AG, Pride NB. Topical corticosteroids potentiate mucin secretion in the normal nose. *Clin Otolaryngol* 21: 76–79, 1996.
- Meltzer EO. Nasal cytological changes following pharmacological intervention. *Allergy* 50: 15–20, 1995.
- Mucignat-Caretta C. Modulation of exploratory behavior in female mice by protein-borne male urinary molecules. *J Chem Ecol* 28: 1853–1863, 2002.
- Mucignat-Caretta C, Bondi M, Caretta A. Animal models of depression: olfactory lesions affect amygdala, subventricular zone, and aggression. *Neurobiol Dis* 16: 386–395, 2004.
- Nava S, Compagnoni ML. Controlled short-term trial of fluticasone propionate in ventilator-dependent patients with COPD. *Chest* 118: 990–999, 2000.
- Phillipps GH. Structure-activity relationships of topically active steroids: the selection of fluticasone propionate. *Respir Med* 84: 19–23, 1990.
- Sergejeva S, Tomaki M, Pullerits T, Zhao LL, Johnson M, Loetvall J. Intranasal fluticasone propionate inhibits allergen induced bone marrow eosinophilia in mice. *Pulm Pharmacol Ther* 15: 129–134, 2002.
- Shepherd GM, Chen WR, Willhite D, Migliore M, Greer CA. The olfactory granule cell: from classical enigma to central role in olfactory processing. *Brain Res Rev* 55: 373–382, 2007.
- Sherrington CA, Mallol J. Early effects of inhaled steroids on airway hyperactivity and pulmonary function in asthma. *Pediatr Pulmonol* 27: 376–382, 1999.
- Silva PL, Passaro CP, Cagido VR, Bozza M, Dolhnikoff M, Negri EM, Morales MM, Capelozzi VL, Zin WA, Rocco PR. Impact of lung remodelling on respiratory mechanics in a model of severe allergic inflammation. *Respir Physiol Neurobiol* 160: 239–248, 2008.
- Sly PD, Collins RA, Thamrin C, Turner DJ, Hantos Z. Volume dependence of airway and tissue impedances in mice. *J Appl Physiol* 94: 1460–1466, 2003.
- van Aalderen WM, Price D, De Baets FM, Price J. Beclomethasone dipropionate extrafine aerosol versus fluticasone propionate in children with asthma. *Respir Med* 101: 1585–1593, 2007.
- Wagers S, Lundblad L, Moriya HT, Bates JH, Irvin CG. Nonlinearity of respiratory mechanics during bronchoconstriction in mice with airway inflammation. *J Appl Physiol* 92: 1802–1807, 2002.
- Witt M, Woźniak W. Structure and function of the vomeronasal organ. *Adv Otorhinolaryngol* 63: 70–83, 2006.
- Xisto DG, Farias LL, Ferreira HC, Picanco MR, Amitrano D, Lapa e Silva JR, Negri EM, Mauad T, Carnielli D, Silva LF, Capelozzi VL, Faffe DS, Zin WA, Rocco PR. Lung parenchyma remodeling in a murine model of chronic allergic inflammation. *Am J Respir Crit Care Med* 171: 829–837, 2005.