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Reduced innervation in the human pharynx in patients with obstructive sleep apnea

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Summary. Obstructive sleep apnea is a disease characterized by repetitive breathing during sleep that lead to reduced oxygen saturation and sleep disturbance among other symptoms. Obstructive sleep apnea is caused by blockade of the upper respiratory airway, although the pathogenic mechanism underlying this occlusion remains unknown. In these studies we explored the hypothesis that alterations in the innervation, especially mechanosensory innervation, of the pharynx may contribute to obstructive sleep apnea. We tested this hypothesis by analyzing the innervation of the human pharynx in normal individuals and in subjects clinically diagnosed with obstructive sleep apnea. Using immunohistochemistry for axon and Schwann cells, as well as for two putative mechanoproteins (ASIC2 and TRPV4), we observed a significant reduction in the density of nerve fibers in the submucosa of patients with obstructive sleep apnea as well as morphological abnormalities in mechanosensory corpuscles. Importantly, while ASIC2 and TRPV4 expression was regularly found in the axons of mechanosensory corpuscles distributed throughout the muscular layer in the control subjects, it was absent in patients with obstructive sleep apnea. These findings support that neurological alterations are important contributors to the pathogenesis of obstructive sleep apnea.

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Introduction

Airway size and resistance are tightly regulated by neural mechanisms in the upper airways (UA) that control muscles and reflexes. The sensory receptors in the UA respond to changes in airway pressure, airflow and temperature, as well as muscle tone. These afferent inputs reach the brainstem respiratory centers, which control UA muscles via efferent motor neural outputs (reviewed by Horner, 2012).

Obstructive sleep apnea syndrome (OSAS; Dempsey et al., 2010) is a common disease that afflicts 2-4% of the population and has a strong genetic component (Paiva and Attarian, 2014). Moreover, it is well known that age, gender, craniofacial structure and the anatomy of the UA, endocrine condition and obesity, but not ethnicity, are associated with OSAS (Ralls and Grigg-Damberger, 2012).

In recent years multiple studies have demonstrated altered UA sensory input and abnormal UA motor function in patients with OSAS using a variety of neurophysiological and histological approaches (Guilleminault et al., 2005; Guilleminault and Ramar, 2009; Saboisky et al., 2012a,b). Association between OSAS and sensory neuropathy outside the UA (Lüdemann et al., 2001), type 2 or type 1A diabetic neuropathy, and axonal subtypes of Charcot-Marie-Tooth disease (Tahrani et al., 2012; Aurora and Punjabi, 2013; Boentert et al., 2013; Fujihara et al., 2013) has

been also demonstrated. Furthermore, it has been hypothesized that local nerve lesions due to long-standing snoring vibrations could be at the basis of OSAS or its progression (Friberg et al., 1998; Friberg, 1999; Svanborg, 2001). Motor neuron lesions and/or direct damage in the muscles (Friberg, 1999; Sunnergren et al., 2011) are also present in OSAS patients. However, the changes in muscle fibers (Lindman and Stål, 2002; Svanborg, 2005; Stål and Johansson, 2012) are not a major contributing factor to OSAS pathogenesis in most patients (Eckert et al., 2007). Thus, there is much evidence that UA neuromuscular abnormalities are frequent in OSAS patients, and all together support the "neurogenic theory" of OSAS (see for a review Saboisky et al., 2012a; Tsai et al., 2013). Consistent with the above data, studies on the innervation of the palate-pharyngeal region in OSAS patients have revealed both increased and decreased number of nerves in the mucosa and muscles (Friberg et al., 1997; Boyd et al., 2004; De Bellis et al., 2012), as well as ultrastructural degenerative changes in myelinated and unmyelinated nerve fibers (Bassiouny et al., 2009).

In the present study we explored the hypothesis that alterations in this mechanosensory innervation may lead to OSAS by analyzing the innervation of the human pharynx in subjects clinically diagnosed with OSAS. The findings support the neurogenic origin paradigm for OSAS (Guilleminault et al., 2005; Guillaumet and Ramar, 2009; Levy et al., 2012).

Materials and methods

Material

The study was carried out using pharynx samples from autopsies of 10 patients that died due to pathologies other than those involving upper respiratory tract or respiratory pathologies. The subjects included in this study were 3 females (age range 47 to 66 years) and 7 males (age range 52 to 78 years) recruited during a 3 year period in the Surgical Pathology Department, Asturias Central University Hospital (HUCA), Oviedo, Spain (Table 1). The subjects selected had been diagnosed with OSAS for more than 2 years according to the criteria of the Spanish Society of Respiratory Pathology (Consensus Document; Puertas Cuesta et al., 2005). As controls, samples from pharynx were obtained during removal of organs for transplantation from subjects that died in traffic incidents (HUCA) from 4 females (age-range 36 to 62 years), and 3 males (age-range 26 to 60 years) free of known OSAS, UA pathologies or neurological disease. All materials were obtained in compliance with Spanish Laws, and the guidelines of the Helsinki Declaration II.

From each pharynx the lateral and posterior walls of the oropharynx and the lateral walls of the hypopharynx were sampled. The tissue samples consisted of 1-2 cm^2 fragments containing the whole thickness of the pharyngeal walls. The samples were fixed in 10% formaldehyde in 0.1 M phosphate buffer saline (PBS) at pH 7.4 for 24 h at 4°C, dehydrated and embedded in paraffin. Sections 10 μm and 30 μm thick perpendicular or parallel to the pharynx surface were obtained, mounted on gelatin-coated microscope slides and processed for immunohistochemistry.

Immunohistochemistry

Indirect peroxidase-antiperoxidase immunohistochemistry was performed as follows: sections were deparaffinized and rehydrated, then rinsed in 0.05 M HCl Tris buffer (pH 7.5) containing 0.1% bovine serum albumin and 0.1% Triton X-100. Thereafter the endogenous peroxidase activity (3% H_2O_2) and non-specific binding (10% fetal calf serum) were blocked, and the sections incubated overnight in a humid chamber at 4°C with primary antibodies (Table 2). The antibody against NSE was used as specific axon marker; antibodies against S100 protein were used to immunolabel Schwann cells and Schwann-related cells (see Vega et al., 2009). Antibodies against ASIC2 and TRPV4 were used to detect these two putative mechanoproteins (Del Valle et al., 2012). After incubation with the primary antibodies, sections were

Table 1. Data about the OSAS subjects used in these studies.

Case number	Age	Gender	Snoring	BMI	Associated diseases	Cause of death
1	47	F	Yes	> 30	No	CVA
2	52	M	Yes	> 30	Hypertension	CVA
3	53	F	Yes	> 20	Type 2 diabetes	CVA
4	61	M	n.d.	> 30	Hypertension	CVA
5	66	F	Yes	> 25	No	DA
6	68	M	Yes	> 30	Hypertension	CVA
7	72	M	Yes	> 30	Hypertension	CVA
8	76	M	Yes	> 20	Type 2 diabetes	CVA
9	78	M	Yes	> 30	Hypertension	PC
10	78	M	Yes	> 20	Hypertension, type 2 diabetes	CVA

CVA, cerebrovascular accident; DA, domestic accident; PC, prostate cancer.

rinsed in the same buffer and incubated with Dako EnVision System labeled polymer-HR anti-rabbit IgG or anti-mouse IgG (DakoCytomation, Denmark) for 30 minutes at room temperature. Finally, sections were washed and the immunoreaction visualized using 3-3'-diaminobenzidine as a chromogen. To ascertain structural details, sections were slightly counterstained with hematoxylin & eosin.

Double immunohistochemistry

Double immunostaining was performed on 10 μm and 30 μm thick deparaffinized and rehydrated sections. Non-specific binding was reduced by incubation for 30 minutes with a solution of 10% bovine serum albumin in TBS. The sections were then incubated overnight at 4°C in a humid chamber with a 1:1 mixture of anti-NSE and anti-S100P antibodies; anti-NSE with anti-ASIC2 or anti-TRPV4 antibodies; anti-S100 with anti-ASIC2 or anti-TRPV4 antibodies. After rinsing with TBS, the

Table 2. Primary antibodies used in the study.

Antigen	Origin	Dilution	Supplier
General neural markers			
NSE (clone BBS/NC/VI-H14)	Mouse	1:1000	Dako[1]
S-100 protein	Rabbit	1:1000	Dako[1]
S100P (clone 4C4.9)	Mouse	1:1000	Thermo Scientific[2]
Mechanoproteins			
ASIC2	Rabbit	1:200	Lifespan Biosciences[3]
TRPV4	Rabbit	1:100	Abcam[4]

ASIC2, acid-sensing ion channel 2; NSE, neuron specific enolase; TRPV4, transient receptor potential channel vanilloid 4; [1]Glostrup, Denmark; [2]Thermo Scientific, Freemont, CA, USA; [3]Seattle, WA, USA; [4]Cambridge, UK. Anti-ASIC2: rabbit polyclonal antibody raised against a synthetic peptide from the extracellular domain of mouse ASIC2 conjugated to an immunogenic carrier protein. Catalogue LS-C93915. Blocking peptide: Lifespan Biosciences LS-PB156. Anti-TRPV4: rabbit polyclonal antibody raised against a synthetic peptide derived from the cytoplasmic N-terminus domain, which is conserved in mouse, human and rat TRPV4, conjugated to immunogenic carrier protein. Catalogue ab63003. Blocking peptide: Abcam ab166832

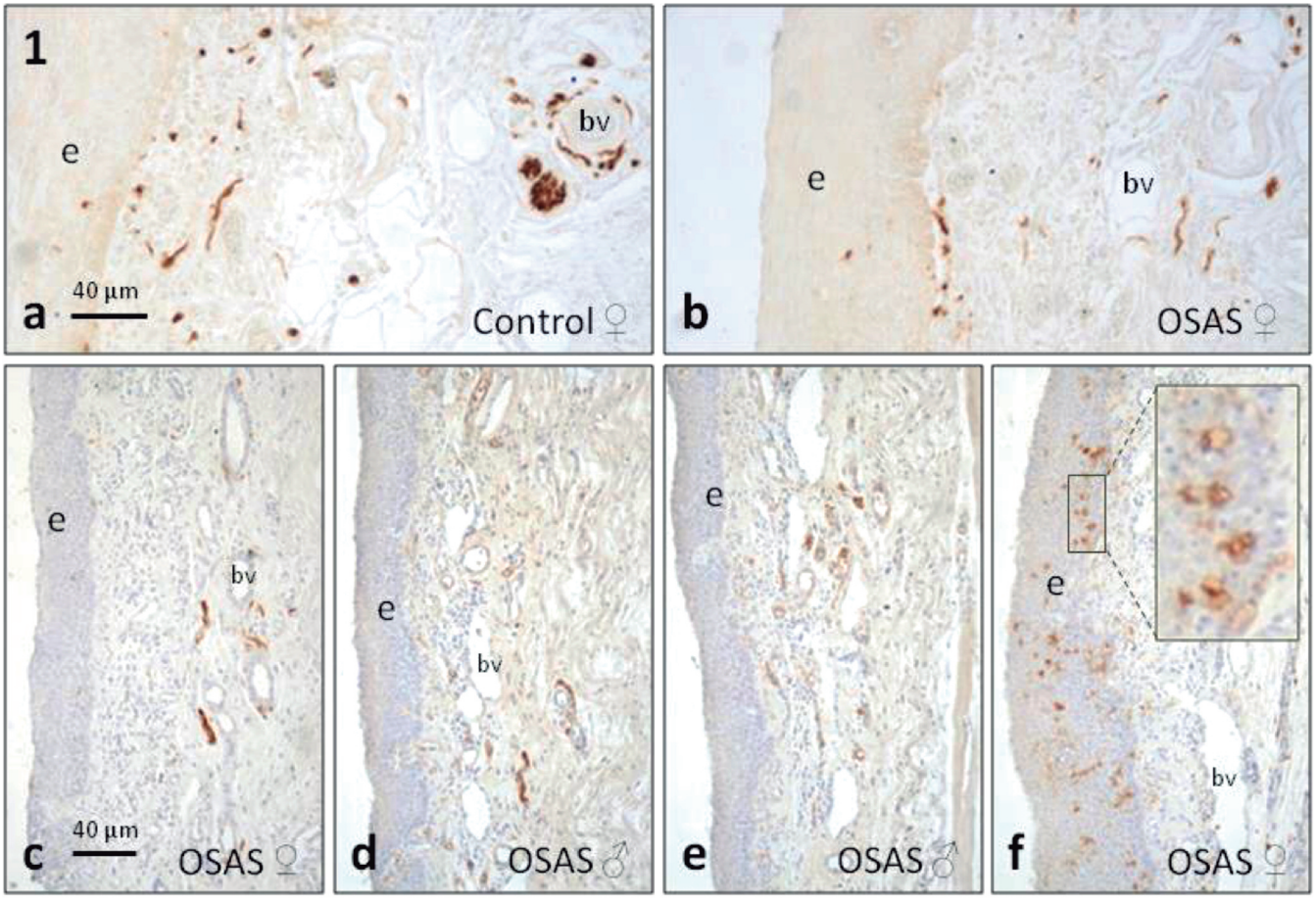

Fig. 1. Submucous nerves in pharynx control **(a)** and OSAS patients **(b-f)** were evidenced using an antibody against the general neuronal marker NSE **(a, b)** and the Schwann cell marker S100 protein **(c-f)**. They form isolated nerve fibers, nerve bundles and perivascular plexuses. In some cases intraepithelial S100 protein positive cells, identified as Langerhans cells, were found. bv, blood vessels; e, epithelium.

sections were incubated for 1 hour with Alexa fluor 488-conjugated goat anti-rabbit IgG (Serotec, Oxford, UK), diluted 1:1000 in TBS containing 5% mouse serum (Serotec), then rinsed again and incubated for another hour with Cy™ 3-conjugated donkey anti-mouse antibody (Jackson-ImmunoResearch, Baltimore, MD, USA) diluted 1:50 in TBS. Both steps were performed at room temperature in a dark humid chamber. Finally, to ascertain structural details, sections were counterstained with DAPI (10 ng/ml). Sections were then washed, dehydrated and mounted with Entellan®. Triple staining was detected using a Leica DMR-XA automatic fluorescence microscope coupled with Leica Confocal Software, version 2.5 (Leica Microsystems, Heidelberg GmbH, Germany) and the images captured were processed using the software Image J version 1.43 g Master Biophotonics Facility, Mac Master University Ontario (www.macbiophotonics.ca).

Controls

To test the specificity of the immunoreactivity representative sections were processed in the same way as described above using non-immune rabbit or mouse sera instead of the primary antibodies, omitting the primary antibodies in the incubation, or using pre-absorbed antibodies for ASIC2 and TRPV4 (5 μg of the blocking peptide in 1 ml of the antibody working solution).

Quantitative study

The density of nerve profiles within the whole pharynx walls, excluding the mucosa, was determined by evaluating the immunoreactive area for S100P and NSE , ASIC2 and TRPV4 in 10 randomly selected fields in 10 sections 200 µm apart using an automatic image analysis system (Quantimet 550, Leika, QWIN Program; Image Analysis Department, University of Oviedo). The evaluation was carried out in duplicate by two independent researchers. The density of nerve profiles was determined using a x4 objective whereas the area occupied by ASIC2 and TRPV4 within the corpuscles was determined using a x20 objective. The values are expressed as mean with standard deviation per 1 mm^2. Statistical differences among groups were analyzed

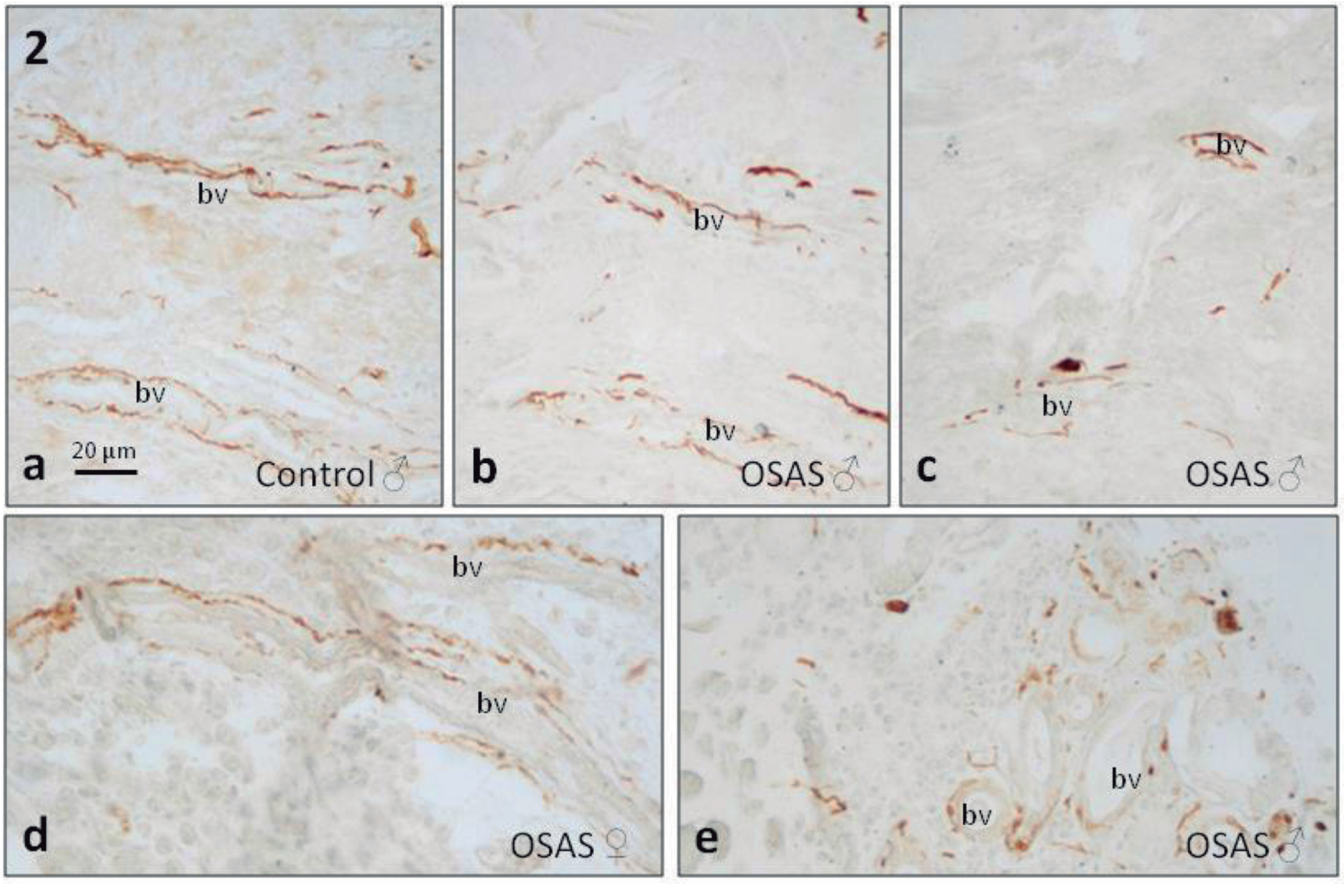

Fig. 2. Perivascular plexuses in pharynx controls **(a)** and OSAS patients **(b-e)** were evidenced using an antibody against the general neuronal marker NSE. bv, blood vessels.

using the Kruskall-Wallis test, and a $p<0.05$ was considered significant.

Results

To identify nerves within the pharyngeal walls we used immunohistochemistry with specific markers for axons and Schwann cells. Nerve profiles were found in all the layers of the pharynx in both control subjects and OSAS patients. Generally, the density of nerve profiles identified was higher with anti-S100 protein antibodies than with anti-NSE antibodies, and higher in the oropharynx than hypopharynx (Table 3; Mu and Sanders, 2000).

Distribution of nerves and sensory corpuscles in the pharynx

The nerves localized in the submucosa, forming perivascular plexuses, or within the muscular layer were quantified automatically; those localized in the pharyngeal adventitia were evaluated only qualitatively. The identification of sensory corpuscles was based on morphological and immunohistochemical criteria, as well as the arrangement of the constituent cells (see Vega et al., 2009).

In control subjects, nerves were observed below the epithelium in close proximity to the basal layer, forming small nerve bundles or dense vascular nerve plexuses (Figs. 1a, 2a). No intraepithelial nerve fibers were found. Within the muscular layer numerous corpuscle-like nerve formations were observed, with a variable pattern of arrangement of axons and Schwann-like cells, and cannot be described following pre-established morphological criteria. They were spiral-wharves shape sensory formations, oval or elongated in shape, Ruffini-like and Meissner-like corpuscles, and tree-like corpuscles (Fig. 3; De Carlos et al., 2013). Most of them were capsulated and supplied by a single axon, branched and showing a very complex arrangement. Double immunostaining confirmed these observations (Fig. 4). Finally, Pacini-like corpuscles were occasionally found in the adventitia and the connective tissue forming the pharyngeal raphe (data not shown).

The pattern described above did not vary qualitatively in the pharynx of OSAS patients, but the density of those structures was clearly reduced.

The innervation of the pharynx is not age- or gender-related.- Gender (higher prevalence in men than in women) and age are two factors to be considered in the pathogenesis of OSAS. In our studies, no gender-related differences were observed in the innervation of the

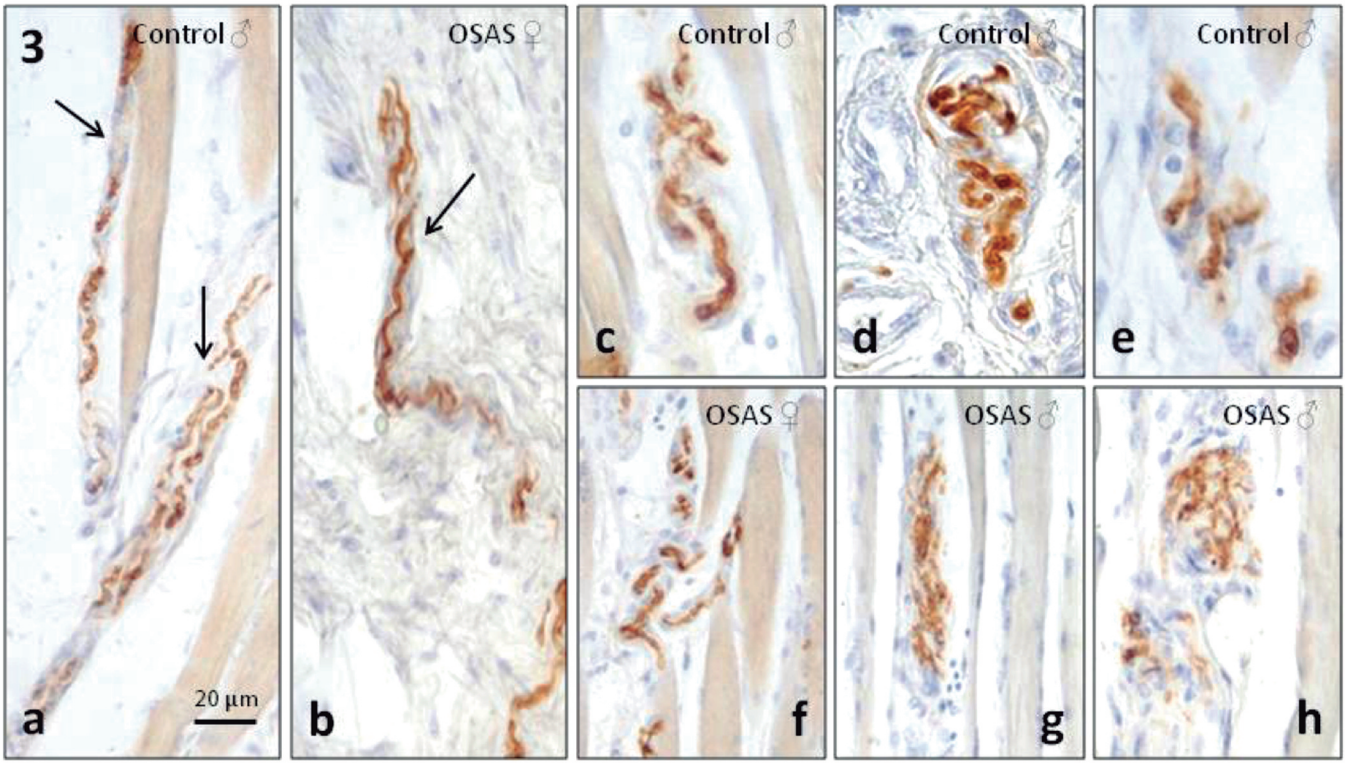

Fig. 3. Different morphotypes of sensory corpuscles in the walls of the human pharynx in control **(a, c-e)** and OSAS **(b, f-h)** subjects. Spiral-wharves nerve structures were regularly found in both groups of subjects **(a and b)**. All the sensory nerve formations identified consisted of axons and Schwann cells, more or less capsulated, and isolated from the surrounding tissues.

Table 3. Area occupied by nerve profiles displaying immunoreactivity for the assessed antigens in the lateral wall of the pharynx of controls and OSAS subjects.

	Controls		OSAS patients	
	Female	Male	Female	Male
Oropharynx				
Submucosa				
S100P	3,7±0,6	3,3±0,4	1,2±0,8[a]	1,4±0,9[a]
NSE	2,9±0,5	2,5±0,3	0,8±0,2[b]	0,7±0,3[b]
ASIC2	nd	nd	nd	nd
TRPV4	nd	nd	nd	nd
Muscular layer				
S100P	6,3±1,6	6,5±0,9	4,2±0,8[a]	4,4±1,0[a]
NSE	5,7±0,8	5,5±0,6	3,7±0,2[a]	3,9±0,6[a]
ASIC2	1,3±0,1	1,1±0,1	0,4±0,1[a]	0,5±0,1[a]
TRPV4	1,1±0,1	1,1±0,2	0,7±0,1[a]	0,7±0,1[a]
Hypopharynx				
Submucosa				
S100P	3,3±0,8	3,6±0,6	1,8±0,2[a]	1,6±0,8[b]
NSE	3,0±0,4	2,8±0,6	1,2±0,4[b]	1,0±0,8[b]
ASIC2	nd	nd	nd	nd
TRPV4	nd	nd	nd	nd
Muscular layer				
S100P	6,0±0,7	5,2±0,9	3,4±0,6[a]	3,4±0,9[a]
NSE	4,9±0,6	4,8±0,3	2,8±0,4[a]	2,9±0,8[a]
ASIC2	0,9±0,1	1,1±0,1	1,0±0,1	0,9±0,1
TRPV4	0,8±0,1	1,0±0,1	1,2±0,4	0,9±0,1

Results are expressed as the percentage of immunoreactive area (mean ± SD) per 1 mm^2. nd, not detectable. [a], p<0,05 vs. the same gender group control; [b], p<0,01 vs. the same gender group control.

Table 4. Correlation between age and density of NSE-positive nerve fibres in the lateral wall of the pharynx of controls and OSAS subjects.

	Controls	OSAS patients
Oropharynx		
Submucosa		
<40 y	2,8±0,5 (n=2)	
40-50 y	3,0±0,9 (n=1)	1,6±0,9 (n=1)[a]
50-60 y	3,0±0,8 (n=4)	1,1±0,7 (n=2)
>60 y		1,3±0,8 (n=7)[a]
Muscular layer		
<40 y	5,3±1,1 (n=2)	
40-50 y	5,5±0,9 (n=1)	3,7±0,8 (n=1)[a]
50-60 y	5,3±0,8 (n=4)	3,1±0,4 (n=2)[a]
>60 y		2,9±0,3 (n=7)
Hypopharynx		
Submucosa		
< 40 y	2,7±0,3 (n=2)	
40-50 y	2,9±0,5 (n=1)	1,3±0,3 (n=1)[a]
50-60 y	2,9±0,2 (n=4)	0,9±0,2 (n=2)[b]
>60 y		0,9±0,3 (n=7)
Muscular layer		
< 40 y	5,1±1,2 (n=2)	
40-50 y	4,8±0,7 (n=1)	3,0±0,3 (n=1)[a]
50-60 y	5,0±0,9 (n=4)	3,3±0,2 (n=2)[a]
>60 y		2,9±0,6 (n=7)

Results are expressed as the percentage of immunoreactive area (mean ± SD) per mm^2. a, p<0,05 vs control of the some age-group.

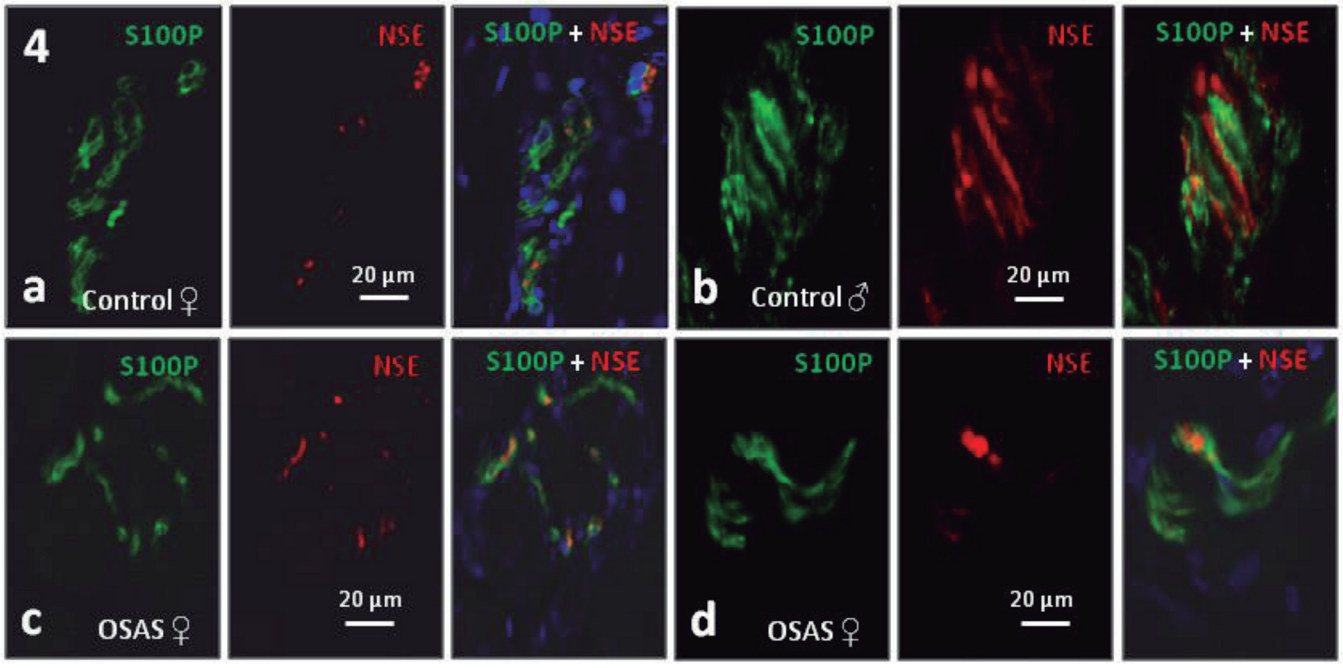

Fig. 4. Sensory formations, or corpuscles from the human pharynx labeled with anti-S100 protein antibody conjugated with Alexa fluor 488 (green fluorescence), and with anti-NSE antibody conjugated with CyTM3 (red fluorescence). The images show the relation between axons and Schwann-related cells in these sensory structures.

oropharynx or the hypopharynx wall layers in control subjects (Table 3). Regarding age, the density of nerves in the pharyngeal layers was similar in control subjects for the range studied (Table 4).

The innervation of the pharynx is reduced in OSAS patients.- After demonstrating that the density of nerves in the pharynx remains constant with age and was independent of gender in normal subjects, the next step of our research was to determine whether or not the pattern of innervation and nerve density is affected in OSAS. Interestingly, the results were rather homogeneous, and as occurred with control subjects, no age-related or gender-related changes were observed in patients with OSAS (Tables 3, 4).

The density of nerve profiles observed in the submucosa of OSAS patients was significantly reduced compared with controls (Fig. 1b-f; Table 3). There was a significant reduction of the submucous nerves and most of the perivascular plexuses were missing or severely reduced. In some cases (3/10) the epithelium of the oropharynx contained a large number of S100 protein positive cells, which, based on their morphology and localization, were identified as Langerhans cells (see De Melo et al., 2006). Of particular interest was the reduction of the perivascular plexuses, which in some cases were completely absent (Fig. 2a-e).

Overall, there was a significant reduction in the innervation density of the muscular layer in OSAS compared with controls, while within the OSAS group no age-related or gender-related changes were detected (Fig. 5; Tables 3, 4). Regarding sensory corpuscles, all the morphotypes found in controls were encountered in OSAS patients, but most of them were smaller and showed a hypothrophic or atrophic aspect (Figs. 3b,f-h, 4c,d).

ASIC2 and to a lesser extent TRPV4 are severely reduced in patients with OSAS

ASIC2 and TRPV4 are two putative mechanoproteins localized in the axon of sensory corpuscles (Del Valle et al., 2012) including those of the human pharynx (De Carlos et al., 2013). The localization of ASIC2 and TRPV4 was investigated using

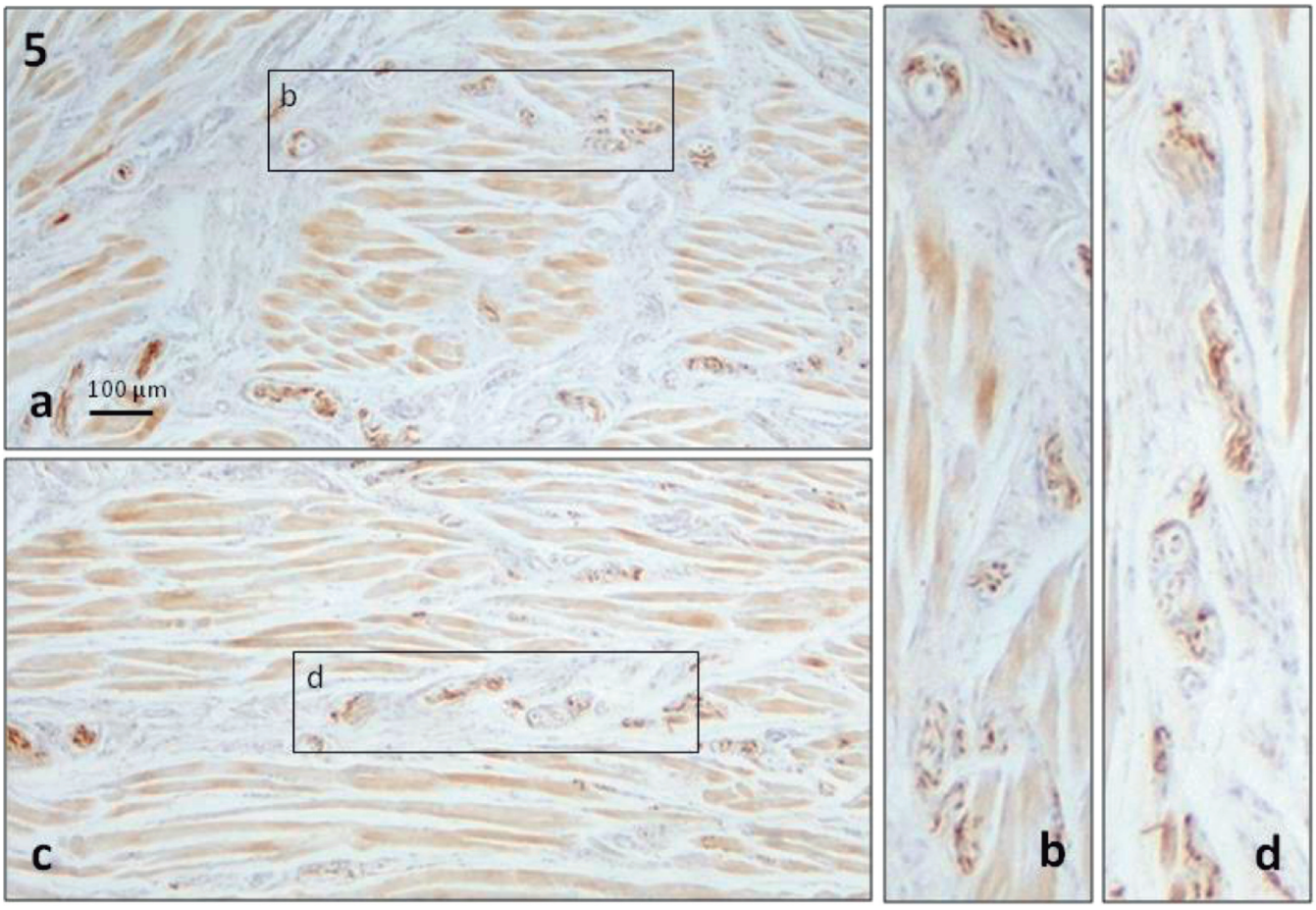

Fig. 5. Low power magnification of nerve apparatus of the muscular layer of control **(a, b)** and OSAS **(c, d)** subjects immunolabelled with NSE.

immunofluorescence and double immunohistochemistry combined with axonic or Schwann cell markers. In control subjects immunoreactity for ASIC2 and TRPV4 was observed in the axons of mechanosensory corpuscles distributed throughout the muscular layer in the control subjects (Fig. 6a,b); these structures were never or only at residual levels detected in OSAS patients (Fig. 6c-e; Table 3) in the oropharynx while no significant changes were found in the hypopharynx. On the other hand, co-localization of both ASIC2 and TRPV4 in the axons strongly supports the mechanosensory function of these formations.

Discussion

The goal of these studies was to investigate the pattern and density of innervation in the human pharynx of patients suffering OSAS. We quantified the density of nerve profiles in the submucosa and muscular layers (mainly formed by the superior constrictor pharyngeal muscle, SCPM), and describe the morphology and mechanoprotein-content of pharyngeal sensory corpuscles as a means to test the neurogenic origin paradigm of OSAS (Guilleminault et al., 2005; Guillaumet and Ramar, 2009; Lévy et al., 2012).

We have not been able to detect the presence of intraepithelial nerve fibres, neither in control nor in OSAS subjects, as reported in other species, but the pattern of nerve distribution in the submucosa and perivascular plexuses is in agreement with previous studies (see for a review and references De Carlos et al., 2013). Recently, we have described the mechanosensory innervation pattern in the human pharynx (De Carlos et al., 2013). Those findings are consistent with the results reported here in control subjects. Furthermore, our findings also demonstrated that there are no age- or gender-related differences in the pattern of innervation of the pharynx within the range analysed.

The collapse of UA during sleep is the hallmark of OSAS (see Eckert and Malhotra, 2008). Two theories have been proposed to explain the pathophysiology of OSAS: the obstructive theory, which postulates that muscle hypertrophy leads to airway narrowing; and the neurogenic theory, which postulates peripheral nerve

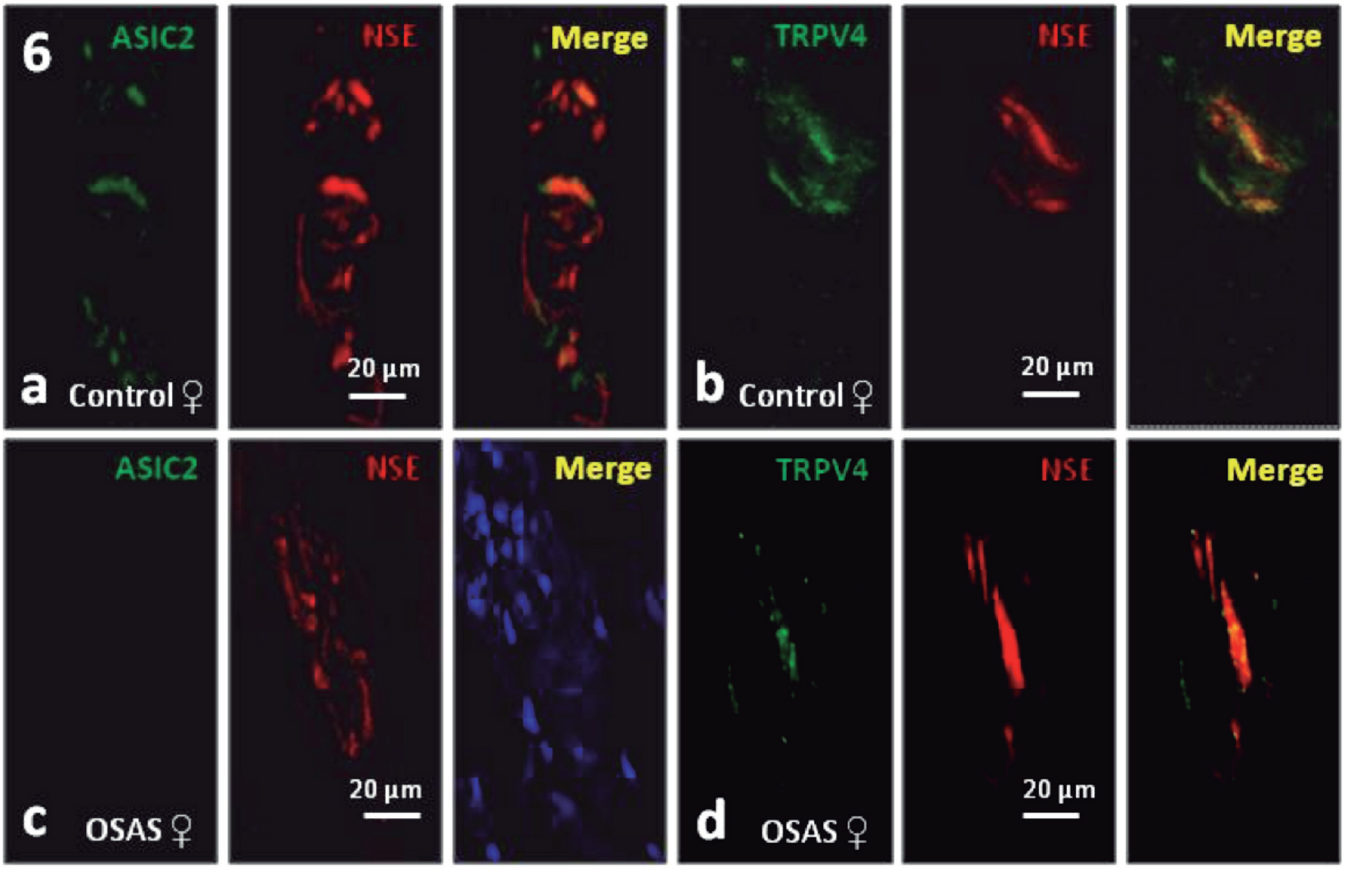

Fig. 6. Immunohistochemical detection of ASIC2 **(a)** and TRPV4 **(b)** in different kinds of mechanoreceptors in the walls of the human pharynx in control subjects **(a, b)** and OSAS patients **(c, d)**. Co-localization studies demonstrate that ASIC2 and TRPV4 (green fluorescence) are primarily found in the axons (NSE; red fluorescence), and were absent (or present at residual levels) in OSAS patients.

degeneration due to vibratory stretch trauma, or systemic diseases, that leads to muscle atrophy and collapse (see Saboisky et al., 2012a; Tsai et al., 2013).

In patients with OSAS there are changes in sensation, muscle properties, and neural tone in the UA; these changes are referred to as airway remodeling. But whereas the muscle fibers, motor nerve fibers and motor endplates of OSAS patients have been studied in depth (Lindman and Stal, 2002; Boyd et al., 2004; Saboisky et al., 2012a), the potential role of sensory nerve impairment in OSAS has not been sufficiently investigated (Kimoff et al., 2001; Nguyen et al., 2005).

We believe that if UA motor axons, anatomically deeper, are affected by vibration, the sensory afferents, closer to the airway surface, should also be impaired. In this study we demonstrate abnormal sensory innervation of the pharynx that can result in diminished reflexogenic dilation of UA mediated by afferents in the pharyngeal mucosa. Our results demonstrate a significant reduction in the density of nerve fibers in the submucosa, muscular layer and perivascular plexuses in OSAS patients, as well as abnormal mechanosensory corpuscles in the muscular layer, which all together support to the neurogenic theory of OSAS. These data are consistent with findings by Bassiouny et al. (2009) and De Bellis et al. (2012) in the human uvula of apneic snorers, but differs from studies of others that have found increased numbers of nerve fibers in the mucosa and muscles (Friberg et al., 1997; Boyd et al., 2004). Importantly, since the markers used are not specific for motor nerve fibers of motor endplates we cannot support motor impairment, although, as a whole, the innervation of the muscular layer was found to be reduced in OSAS.

Regarding mechanosensory corpuscles in the muscular layer, the primary goal of those studies, they were readily detectable in OSAS patients, but they were disarranged and smaller than in control subjects. Furthermore, there was an almost complete loss of ASIC2 and TRPV4 in the oropharyngeal mechanoreceptors of OSAS subjects. Overall, these findings suggest that patients with OSAS have impaired pharyngeal sensitivity and mechanosensation. The proprioception arising from pharyngeal muscles is important in the physiology of the UA for regulation of important reflexes related to normal breathing (see for a review Miller, 2002). Interestingly, patients with OSAS have altered vibration and cold detection thresholds (Larsson et al., 1992; Hagander et al., 2009).

Some limitations of the present study were the small number of subjects and that the biopsies were obtained from a limited area of the AU that may not be representative of the whole organ. It is likely that the absence of age-related and gender-related changes in OSAS patients is related to the small number of subjects included in the study (Eikermann et al., 2007; Ralls and Grigg-Damberger, 2012). Further studies are now in progress in our laboratory sampling larger areas of the palatum and pharynx in order to confirm whether the changes in innervation reported here involve the whole UA.

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