

Neurotrophic Factor BDNF Is Upregulated in Soft Palate Muscles of Snorers and Sleep Apnea Patients

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Objectives: Neuromuscular injuries are suggested to contribute to upper airway collapse and swallowing dysfunction in patients with sleep apnea. Neurotrophins, a family of proteins involved in survival, development, and function of neurons, are reported to be upregulated in limb muscle fibers in response to overload and nerve damage. We aimed to investigate the expression of two important neurotrophins, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), in muscle fibers of uvula from snorers and sleep apnea patients and to compare these findings with pharyngeal function.

Methods: Uvula muscle biopsies from 22 patients and 10 controls were analyzed for BDNF, NGF, and cytoskeletal protein desmin using immunohistochemistry. Pharyngeal swallowing function was assessed using videoradiography.

Results: BDNF, but not NGF, was significantly upregulated in a subpopulation of muscle fibers in snoring and sleep apnea patients. Two major immunoreaction patterns for BDNF were observed; a fine grainy point like BDNF staining was displayed in muscle fibers of both patients and controls (41 ± 23 vs. $25 \pm 17\%$, respectively, $P = .06$), while an abnormal upregulated intense-dotted or disorganized reaction was mainly observed in patients (8 ± 8 vs. $2 \pm 2\%$, $P = .02$). The latter fibers, which often displayed an abnormal immunoreaction for desmin, were more frequent in patients with than without swallowing dysfunction (10 ± 8 vs. $3 \pm 3\%$, $P = .05$).

Conclusion: BDNF is upregulated in the upper airway muscles of snorers and sleep apnea patients, and especially in patients with swallowing dysfunction. Upregulation of BDNF is suggested to be a response to denervation, reinnervation, and repair of injured muscle fibers. Our findings propose that damaged upper airway muscles might heal following treatment for snoring and sleep apnea.

Level of Evidence: NA

Key Words: Neurotrophins, brain-derived neurotrophic factor (BDNF), nerve-derived neurotrophic factor (NGF), snorers, obstructive sleep apnea, OSA, swallowing dysfunction, desmin, neuromuscular injury, nerve, muscle fiber.

INTRODUCTION

Obstructive sleep apnea is a prevalent disorder characterized by repetitive collapse of the upper airway during sleep followed by transient hypoxia and over a period an increased risk for cardiovascular disease including stroke and hypertension.¹⁻³ The pathophysiology behind the upper airway collapse remains unclear, but it is suggested that traumatic snoring vibrations cause neuromuscular injuries leading to inefficient muscle function during sleep.⁴⁻⁸ Changes typical of neuromuscular injuries have been reported in the upper airway muscles of snoring and

obstructive sleep apnea patients.⁹⁻¹² Moreover, swallowing dysfunction is common among these patients.¹³⁻¹⁵

Tissue damage evokes a complex process in an attempt to repair. This process involves a cascade of signal substances necessary for normal healing and regeneration. Neurotrophins are a family of proteins capable of signaling to nerve cells for their survival, differentiation, or growth.¹⁶ Limb muscle fibers have been reported to express neurotrophins and their receptors not only in the developmental stages,¹⁷⁻¹⁹ but also during muscle transformation as well as in various physiological and pathological conditions.²⁰ The presence of neurotrophins in several non-neuronal cells suggests their wider role apart from the nervous system.^{21,22} The presence of neurotrophins in upper airway muscle fibers has not been investigated previously.

We hypothesize that neurotrophins are upregulated in upper airway muscle fibers of snorers and sleep apnea patients in response to local neuromuscular injuries caused by traumatic snoring vibrations and muscle overload. We therefore aimed to explore the expression patterns of two well-known neurotrophic factors, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in soft palate muscle fibers of snorers and sleep apnea patients. The outcome was compared with deviations in pharyngeal swallowing function.

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MATERIALS AND METHODS

Patients and Controls

Twenty-two patients (one female, 21 males), mean age 45 years (range 29–60), referred for soft palate surgery either because of long-term habitual snoring or snoring with sleep apnea, were included. Exclusion criteria were smoking, previous palatal surgery, systemic disease, medications, and drug abuse. Ten healthy volunteering male controls, mean age 38 years (range 30–51), were recruited through local advertisement. Exclusion criteria were similar as in patients but also included habitual snoring and sleep apnea. Both patients and voluntary controls underwent overnight sleep apnea recordings and videoradiographic examination of swallowing function.

Ethical Approval

The regional Medical Ethical Committee at Umeå University (Dnr 05-130 M) approved the study protocols, and patients and voluntary controls gave their written consent to participate. All muscle samples were collected in agreement with the Declaration of Helsinki.

Sleep Apnea Recordings

A portable monitoring device was used for recordings of sleep apnea (Embletta, Embla systems, Kanata, Canada). The device recorded continuous signals for airflow using nasal cannula pressure, thoracic and abdominal respiratory movements, finger pulse-oximetry (Nonin Oximeter Xpod, Nonin Medical, Inc., Plymouth, MN, USA) and body position. Recorded data was manually scored according to the American Academy of Sleep Medicines recommendation. The definition of an apnea was a $\geq 90\%$ cessation of airflow lasting at least 10 seconds, and a hypopnea was defined as 50% reduction in airflow compared with baseline, in combination with an oxygen desaturation of $\geq 3\%$.²³

Swallowing Examination

Swallowing function was videoradiographically examined (C-arm, Philips BV 29, field width 23 cm) in an upright position and with lateral and frontal projections. The subjects first swallowed a solid bolus including barium sulphate (Mixobar Esophagus; Astra) and then a liquid barium sulphate contrast bolus (Mixobar High Density; Astra). All standard boluses were repeated twice in each projection. Two investigators examined the recordings. Pharyngeal swallowing function was graded as normal function or dysfunction. Swallowing dysfunction was present if one of the following deviant features appeared; premature leakage, velar dysfunction, residual or laryngeal penetration, dysfunction of the upper esophageal sphincter or the epiglottis.²⁴

Questions Related to Snoring

Patients answered a questionnaire how often they snored loud and duration in years of snoring. The frequency of loud snoring was graded on a 1 to 5 scale: 1) never, 2) seldom, 3) sometimes, 4) often, 5) very often.

Acquisition of Tissue Samples

The base of uvula was acquired following soft palate surgery. In controls, a sample from the corresponding area of the uvula was obtained by punch biopsy technique from nine controls

and in one case by complete surgical resection of the uvula. The biopsies were taken under general or local anesthesia.

Biopsy Processing

The biopsies were oriented and fixed using 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0, for 24 h at 4 °C. After fixation, the samples were washed overnight at 4 °C in Tyrodes solution containing 10% sucrose and then mounted for transverse sectioning in OCT compound (Tissue Tek, Miles, Elkhart, IN, USA). Subsequently, samples were frozen in liquid propane chilled with liquid nitrogen and stored at –80 °C until further processing.

Staining for Basic Histology

Using a cryostat (Leica CM 3050), biopsies were cut at –20° into 7- to 8- μ m thick cross-sections and mounted on glass slides. For demonstration of basic morphology, the sections were stained with routine hematoxylin–eosin (H&E).

Immunohistochemistry

Five- μ m thick cross-sections, serial to those above, were cut and mounted on chrome-alum gelatin pre-coated glass slides. Immunohistochemical staining was performed using modified standard techniques and characterized monoclonal (mAb) and polyclonal (pAb) antibodies. In brief, the sections were immersed in 5% normal non-immune donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 15 min and rinsed in 0.01 M phosphate-buffered saline (PBS) for 3 x 5 min. The sections were then incubated with the primary antibody diluted to appropriate concentrations in PBS with bovine serum albumin in a humid environment. Incubation was carried out overnight at 4 °C. After additional washes in PBS, the sections were immersed in 5% normal non-immune donkey serum for 15 min. Thereafter the sections were incubated with secondary antibody (37 °C for 30 min). The sections were then washed in PBS for 3 x 5 min and mounted in Vectashield Mounting Medium (H-1000) or Mounting Medium with 4'6-diamidino-2-phenylindole (DAPI) for staining of nuclei (Vector Laboratories, Burlingame, CA, USA). Bound primary antibodies were visualized by indirect immunofluorescence.

Primary Antibodies

The utilized primary polyclonal antibodies were directed against BDNF (code: sc 546, dilution: 1:1000) and NGF (code: sc 548, dilution 1:2000), both from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The monoclonal antibody against intermediate filament protein desmin (code: M0760, dilution: 1:1000) from DAKO (Glostrup, Denmark) was used as a marker for myofibrillar integrity. All the used antibodies have previously been well characterized for their specificity.^{25–31}

Secondary Antibodies

For BDNF and NGF immunostainings, donkey anti-rabbit Fluorescein (FITC) (1:100) was used as secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). For demonstration of desmin, immunostainings with donkey anti-mouse Alexa fluor 488 (1:300) (Invitrogen Corporation, CA, Carlsbad, USA) or donkey anti-mouse Rhodamine Red-X (1:500) (RRX; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used.

Control Staining and Pre-Absorption

For specificity of staining activity, sections were treated as above except that the primary antibodies were excluded. Furthermore, to confirm the specificity of the antibodies against BDNF and NGF, the antibodies were pre-absorbed with BDNF (50 µg/mL antiserum: sc-546P, Santa Cruz) and NGF (50 µg/mL antiserum; sc-548P, Santa Cruz) peptides. The pre-absorption was done overnight at 4 °C.²⁹

Analysis of Muscle Fibers Expressing BDNF Immunoreactivity

For analysis and quantification of muscle fibers showing immunoreaction for neurotrophins, four randomly chosen muscle areas in uvula were scanned at 40x magnification with a fluorescence/light microscope (Leica DM6000B; Leica Microsystems CMS GmbH, Wetzlar, Germany). The microscope was equipped with a color charged couple device (CCD) camera (Leica DFC490) and a digital high-speed fluorescence CCD camera (Leica DFC360 FX). In each photograph, the total number of muscle fibers was counted manually, and the percentage of muscle fibers showing immunoreaction for neurotrophins was calculated. The percentage of neurotrophin-positive fibers in each individual was calculated from four photos and the values for the uvula muscle is presented as the mean percentage (±SD) in patients and controls. A total of 3208 muscle fibers in patients (n = 2144) and controls (n = 1064) were evaluated and quantified for expression of neurotrophins. For detailed analysis of neurotrophin expression in individual muscle fibers, photographs scanned at 60x magnification were used.

Two investigators, who were blinded for the origin of the biopsies, analyzed the samples first separately and then together; the final assessment was based on a consensus between them.

Statistical Analysis

Statistical tests were performed with SPSS software (IBM SPSS 23, statistical software). A Shapiro–Wilk test was used to detect normality in the data obtained from findings in muscle fibers. Since the data was non-normally distributed, a non-parametric Mann–Whitney U test was performed. Values are presented as mean proportion ± standard deviation. A *P*-value ≤ .05 was considered significant.

RESULTS

Patients and Controls

All patients snored (mean 20 years, range 6–40), and 14 of the 22 patients had obstructive sleep apnea with a mean apnea hypopnea index (AHI) > 5 (mean AHI 24, range 5–84). All patients reported that they snored loud often or very often (grade 4 or 5). Sixteen patients had swallowing dysfunction and six had a normal function. Mean body mass index (BMI) was 28 kg/m² (range 21–34).

The 10 controls had an AHI index <5, none was habitual snorer and they all displayed a normal swallowing function. Mean BMI for controls was 24 kg/m² (range 22–30).

Morphology of Muscles

Muscle morphology in the uvula of the controls revealed round to polygonally shaped muscle fibers with relatively close proximity to each other (Fig. 1A). In

contrast, the muscles from patients displayed morphology typical of neuromuscular injuries, ie, a high number of hypo- and hypertrophic fibers, and an increased amount of connective tissue (Fig. 1B).

BDNF Expression in Muscle Fibers

Two major immunoreaction patterns for BDNF were observed in a subpopulation of muscle fibers, one displayed an even distribution of a fine grainy point like staining reaction in both patients and controls (Fig. 1C), while the other pattern showed a dotted to disorganized intense reaction mainly observed in muscle fibers of patients (Fig. 1D). The mean percentages of muscle fibers displaying a fine grainy point like staining reaction was 41 ± 23% versus 25 ± 17% in patients and controls, respectively (*P* = .06), whereas the mean percentage of dotted to disorganized intense reaction was 8 ± 8% in patients and 2 ± 2% in controls, respectively (*P* = .02) (Fig. 1E–F). Intense dotted and disorganized reaction for BDNF is further on referred to as abnormally upregulated BDNF expression. Muscle fibers with an abnormally upregulated BDNF expression (Fig. 2B–D) frequently displayed a disrupted or deficient desmin immunoreaction (Fig. 2F–H). In patients, 73 ± 17% of the fibers showing upregulated expression of BDNF had a deficiency of or a disorganized distribution of desmin. In contrast, the muscle fibers showing none or a grainy point like distribution of BDNF had a normal striated immunoreaction for desmin (Fig. 2A, E). No staining reaction for BDNF was observed in muscle fibers after preabsorption (Fig. 3).

BDNF Expression in Muscle Fibers of Patients With and Without Swallowing Dysfunction

Patients with swallowing dysfunction showed a significantly higher proportion of muscle fibers with abnormally upregulated BDNF expression compared to patients without swallowing dysfunction (10 ± 8 vs. 3 ± 3%, *P* = .05) (Fig. 4).

BDNF Expression and Frequency of Loud Snoring and Years of Snoring

There was no significant difference in the mean percentage of muscle fibers expressing abnormal upregulation of BDNF in patients reporting grade 4 or 5 in frequency of loud snoring. No correlation was found between years of snoring and abnormal upregulation of BDNF.

NGF Expression in Muscle Fibers

Although NFG immunoreaction was observed in nerves, no immunoreaction for NGF was observed in muscle fibers of either controls or patients.

DISCUSSION

This study reports that expression of neurotrophin BDNF is abnormally upregulated in muscle fibers of

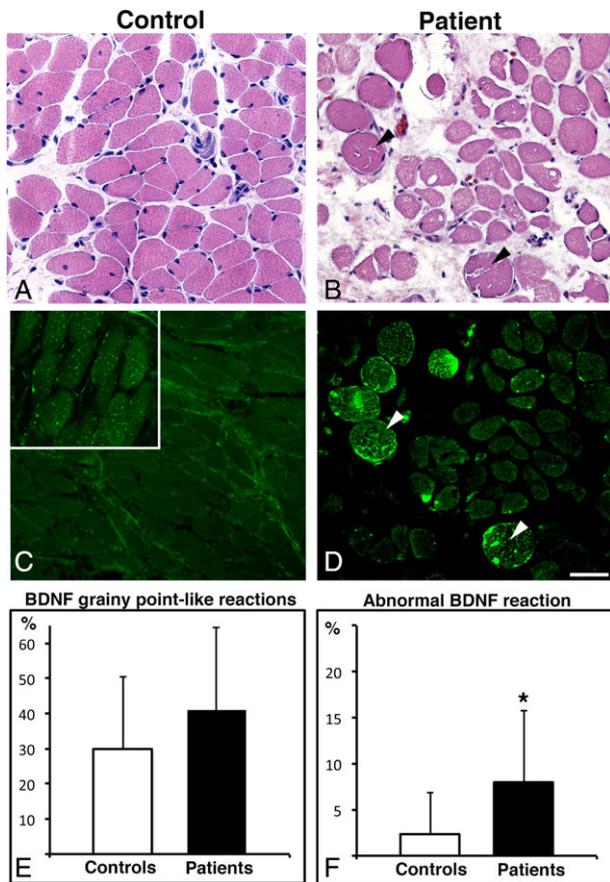


Fig. 1. Morphology and BDNF expression in the uvula muscle of controls and sleep apnea patients. Transverse muscle cross-sections from a control (A and C) and a patient (B and D). Sections stained with H&E (A, B) and for BDNF (C, D). Note increased interstitial connective tissue and fiber size variability in the patient (B) compared to the control (A). Muscle fibers not expressing BDNF in a control are shown in (C). The inset in (C) shows a grainy BDNF reaction in a subgroup of fibers in the control sample. Abnormally upregulated BDNF in muscle fibers (arrowhead) of a patient are shown in (D). Bar graphs display the proportion of grainy point-like (E) and abnormally upregulated BDNF reaction (F) in patients and controls (* $P < .05$). Scale bar 50 μ m.

uvula in snorers and sleep apnea patients and that fibers expressing abnormal upregulation of BDNF were significantly more frequent in patients with swallowing dysfunction. This upregulation of BDNF indicates a dynamic process of reinnervation and repair of injured muscle fibers, probably a consequence of tissue injuries caused by traumatic snoring vibrations and muscle overload. The upregulation of BDNF in pharyngeal muscles gives new insight into the pathophysiology of obstructive sleep apnea and swallowing dysfunction in these patients.

In both controls and patients, an even grainy point like immunoreaction for BDNF was present in a subpopulation of muscle fibers (Fig. 1C). This finding is supported by a previous study, reporting that low levels of BDNF are present along the entire length of myofibrils in healthy unexercised animals.³² Although the role of BDNF in muscle fibers is unclear and may vary under different physiological conditions, a grainy point-like expression of BDNF seems to be a normal biological state in humans. An increase in BDNF mRNA and its expression within skeletal muscle has previously been reported in both human and animal studies in response to various physical exercises, electrical muscle stimulation, and muscle overload.³³⁻³⁶ The presence of BDNF in muscle cells of both patients and controls further support muscles as a secretory organ, producing several myokines such as BDNF.³⁷ Therefore, in accordance with the effect of BDNF in other organs,^{35,38} BDNF could have autocrine, paracrine and trophic effects in the uvula muscles.³⁹ The higher mean proportion of fibers with grainy point-like expression of BDNF in patients ($P = .06$) could be a consequence of higher load on upper airway muscles in snorers and sleep apnea patients.

Muscle fibers in patients displayed an abnormally upregulated BDNF expression in a dotted to disorganized pattern, while this BDNF expression was rare or absent in muscle fibers of controls. Interestingly, muscle fibers displaying upregulated BDNF expression often lacked or had disorganized expression of intermediate filament desmin (Fig. 2). Desmin is a muscle specific cytoskeletal

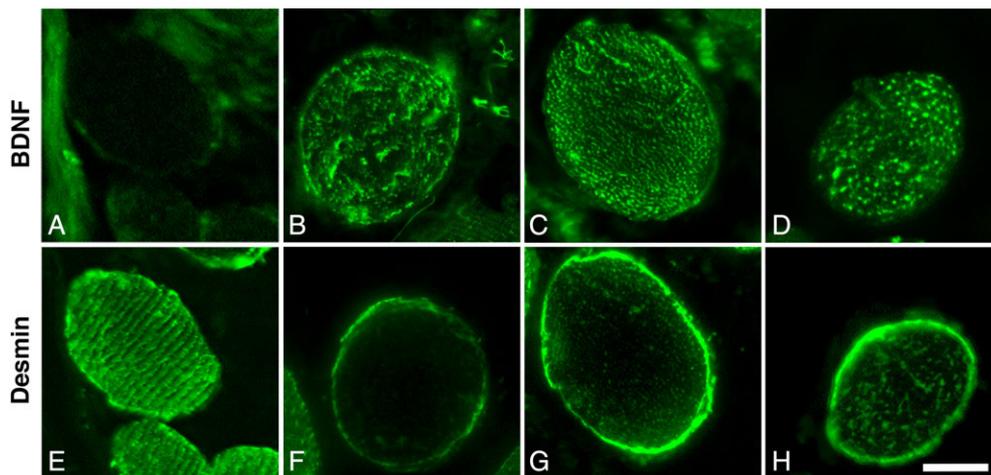


Fig. 2. BDNF and desmin expression patterns in muscle fibers of uvula from sleep apnea patients. Serial muscle transverse sections immunostained for BDNF (A-D) and for desmin (E-H). A muscle fiber without BDNF immunoreactivity (A) and with a normal striated staining pattern characteristically seen for desmin is shown in (E). Muscle fibers with abnormal BDNF expression (B-D), either showing a disorganized (B), a dense point-like (C), or a sparsely distributed dense dot-like pattern (D) in fibers deficient or having a maldistribution of desmin is shown in (F-H). Scale bar 25 μ m.

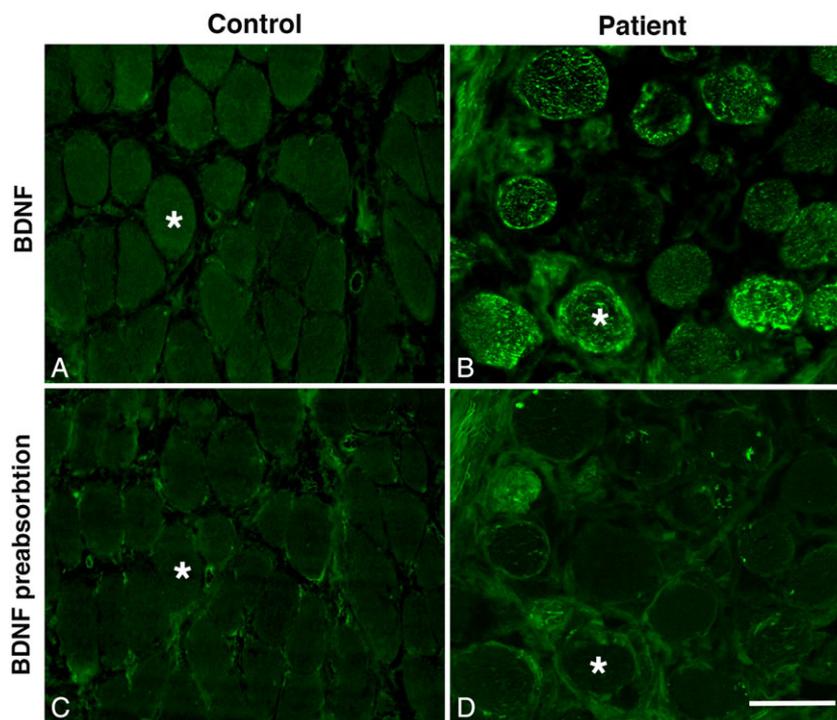


Fig. 3. **Preabsorption with BDNF peptide in a muscle from control and sleep apnea patients.** Transverse sections from a control subject (A, C) and a patient (B, D). Sections are labeled for BDNF (A, B) and for BDNF after preabsorption with a BDNF peptide (C, D). Note the absence of BDNF immunoreaction after preabsorption (C and D). Asterix show corresponding muscle fibers in the control (A, C) and patient (B, D). Scale bar 25 μ m.

protein integrating cellular structures^{40,41} and the absence of this protein in genetic myopathies and animal gene knockout experiments leads to severe and progressive muscle weakness.^{42,43} Changes in the distribution of desmin in muscle fibers have been reported after muscle denervation, insufficient blood supply and acute or eccentric exercise.^{44–46} Since BDNF upregulated in a dotted to disorganized pattern was rarely found in muscles of controls, its presence in patients likely reflect neuromuscular injury or muscle overload.

Sensorimotor neuropathy has been suggested as a cause for the upper airway collapse in sleep apnea patients.^{6,10,47–51} Recently we have shown significant axon and Schwann cell degeneration in nerves of soft palate in snorers and sleep apnea patients.¹⁵ The presence of increased proportion of hypo- and hypertrophic fibers and fibrosis in the uvula muscle from patients observed in this and in previous studies, underscore motor-nerve damage.^{9,10,15} Moreover, following denervation, it is reported that BDNF is expressed in the local environment in an attempt to rescue the motor neurons as well as in the muscle targets of motor neurons.^{38,52} Based on this, muscle fibers with abnormally upregulated BDNF in the soft palate of patients might be related to denervation or reinnervation.

Presence of neuromuscular injuries in the soft palate of snorers and sleep apnea patients are strengthened by the higher proportion of muscle fibers with abnormally upregulated BDNF expression in patients with swallowing dysfunction. Such injuries might also contribute to the inefficiency of muscles to maintain the upper airway

patency during sleep. A plausible mechanism explaining upregulation of BDNF in soft palate muscles from snorers and sleep apnea patients could be an attempt to repair and regenerate injured or denervated muscle fibers following traumatic snoring vibrations and muscle overload.

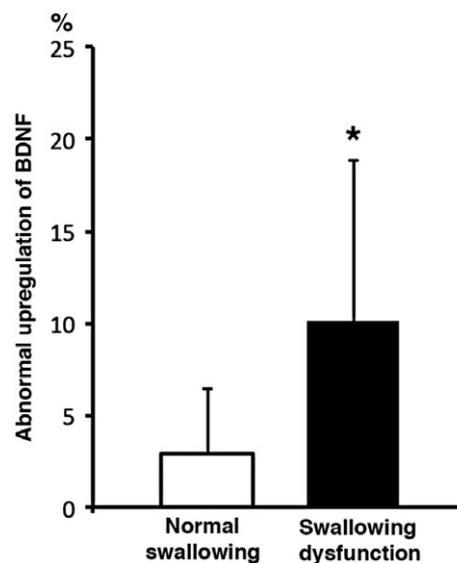


Fig. 4. **Bar graphs showing level of BDNF expression in muscle fibers of patients with or without swallowing dysfunction.** A significantly higher number of fibers with an abnormal upregulation of BDNF in patients with than without swallowing dysfunction (* $P < .05$).

We conclude that BDNF is upregulated in a subpopulation of muscle fibers in the soft palate of snoring and sleep apnea patients and that this upregulation of BDNF was more frequent in patients with swallowing dysfunction. These novel findings reflect muscle overload and neuromuscular injuries as contributing factors to upper airway dysfunction in snoring and sleep apnea patients. Strategies aimed at intervention of traumatic snoring vibration might have beneficial effects on the healing and regenerative process in upper airway muscles of snoring and sleep apnea patients and improve the commonly occurring pharyngeal swallowing dysfunction in these patients.

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The authors declare no conflicts of interest.

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