Research Report

Searching for proprioceptors in human facial muscles

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HIGHLIGHTS

- Craniofacial muscles innervated by the facial nerve lack muscle spindles.
- Proprioceptive acuity in the facial muscles is accurate.
- Proprioception of facial muscles is provided by the trigeminal nerve.
- Putative mechanoproteins are present in mammalian muscle spindles.
- Facial muscles contain putative proprioceptors supplied by mechanosensory nerves.

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ABSTRACT

The human craniofacial muscles innervated by the facial nerve typically lack muscle spindles. However these muscles have proprioception that participates in the coordination of facial movements. A functional substitution of facial proprioceptors by cutaneous mechanoreceptors has been proposed but at present this alternative has not been demonstrated. Here we have investigated whether other kinds of sensory structures are present in two human facial muscles (zygomatic major and buccal). Human checks were removed from Spanish cadavers, and processed for immunohistochemical detection of nerve fibers (neurofilament proteins and S100 protein) and two putative mechanoproteins (acid-sensing ion channel 2 and transient receptor potential vanilloid 4) associated with mechanosensing. Nerves of different calibers were found in the connective septa and within the muscle itself. In all the muscles analysed, capsular corpuscle-like structures resembling elongated or round Ruffini-like corpuscles were observed. Moreover the axon profiles within these structures displayed immunoreactivity for both putative mechanoproteins. The present results demonstrate the presence of sensory structures in facial muscles that can substitute for typical muscle spindles as the source of facial proprioception.

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1. Introduction

The craniofacial muscles are innervated by the facial nerve or the mandibular branch of the trigeminal nerve. Typically the human craniofacial muscles innervated by the facial nerve (refereed here as facial muscles) are devoid of proprioceptors [1–4], i.e. the specialized sensory organs muscle spindles and Golgi's tendon organs [5]. Conversely the trigeminal-innervated craniofacial muscles contain muscle spindles [6,7]. In spite of this, the proprioceptive acuity in the orofacial muscles is more accurate than in the jaw muscles [8], and facial proprioception is important in coordinating facial movement and speech, food, and non-verbal facial communication [9–13]. On the other hand, because the absence of afferent nerve fibers in the peripheral branches of the facial nerve, the putative facial proprioceptors should be innervated by afferent fibers of the trigeminal nerve [14,15] throughout the numerous anastomoses between both nerves [16–18].

To replace the function of facial proprioceptors, some authors have proposed that facial cutaneous mechanoreceptors work as proprioceptors [19,20] but their characteristics and physiological properties suggest they are not the alternative to muscle spindles

[21,22]. Given the absence of typical proprioceptors in facial muscles and the scarce possibility to be functionally substituted by cutaneous mechanoreceptors, we have investigated here whether other kinds of sensory structures are present in human facial muscles. In this way, we have observed recently that pharyngeal muscles, which also lack muscle spindles, contain special morphotypes of mechanoreceptors [23]. Thus, the present study was aimed at investigating the source of facial muscles proprioception, by searching for morphologically atypical proprioceptors in facial muscles. The identification of these structures was based on morphological criteria but also in the expression of putative mechanoproteins (acid-sensing ion channel 2: ASIC2; transient-receptor potential vanilloid 4: TRPV4; see 24) which are present in mechanoreceptors, including muscle spindles [23–28].

2. Material and methods

Samples of the whole cheek thickness containing the buccal and zygomatic major muscles, were obtained from 7 hemi-heads of 7 Spanish cadavers (3 males and 4 females; age range 48–76 years; Section of Anatomy, Departamento de Morfología y Biología Celular, Universidad de Oviedo, Spain). The cadavers were frozen and maintained at $-25°$ C until use. The material was obtained in compliance with Spanish Laws.

The pieces (2,x1,5 × 1,5 cm, approximately) were fixed 48 h in 10% formaldehyde in 0.1 M phosphate buffer saline (PBS) at pH 7.4 for 48 h at 4 °C, washed in tap water for 6 h, and routinely embedded in paraffin. Sections 10 μm thick, perpendicular to the facial skin surface, were obtained, mounted on gelatin-coated microscope slides and processed for immunohistochemistry. Moreover structural hematoxylin & eosin and Masson's trichromic techniques were carried out to ascertain structural details.

Indirect peroxidase peroxidase-antiperoxidase immunohistochemistry was performed as described earlier [23] using the EnVision System kit (Dako, Glostrup, Denmark) following the manufacturer instructions. The primary antibodies used are included in Table 1, and the sections were incubated overnight in a humid chamber at 4 °C. The immunoreaction visualized using 3-3′-diaminobenzidine as a chromogen.

Selected sections were processed for simultaneous detection of NFP and ASIC2, and NFP and TRPV4 as described previously [23]. The sections were then incubated overnight at 4 °C in a humid chamber with a 1:1 mixture of anti-NFP with anti-ASIC2 or anti-TRPV4 antibodies (Table 1). Then the sections were incubated for 1 h with Alexa fluor 488-conjugated goat anti-rabbit IgG (1:1000; Serotec, Oxford, UK), then rinsed and incubated for

Table 1
Primary antibodies used in the study.

Antigen	Origin	Dilution	Supplier
NFP (2F11)	Mouse	Prediluted	Dako[a]
S100P (clone 4C4.9)	Mouse	1:1000	Thermo Scientific[b]
ASIC2	Rabbit	1:200	Lifespan Biosciences[c]
TRPV4	Rabbit	1:100	Abcam[d]

ASIC2: acid-sensing ion channel 2; NFP: neurofilament protein; S100P: S100 protein; TRPV4: transient receptor potential channel vanilloid 4.
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 conserved in mouse, human and rat TRPV4 conjugated to immunogenic carrier protein. Catalogue ab63003. Blocking peptide: Abcam ab166832.

  [a] Glostrup, Denmark.
  [b] Thermo Scientific, Freemont, CA, USA.
  [c] Seattle, WA, USA.
  [d] Cambridge, UK.

another hour with Cy[TM]3-conjugated donkey anti-mouse antibody (1:50; Jackson-ImmunoResearch, Baltimore, MD, USA). Finally, to ascertain structural details sections were counterstained with DAPI (10 ng/ml). Triple fluorescence was detected using a Leica DMR-XA automatic fluorescence microscope coupled with a 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; see also the legend of supplementary material).

For control purposes representative sections were processed in the same way as described above using non-immune rabbit or mouse sera instead of the primary antibodies, or omitting the primary antibodies in the incubation. Moreover, additional experiments using pre-absorbed antibodies for ASIC2 and TRPV4 (5 μg of the blocking peptide in 1 ml of the antibody working solution) were carried out (blocking peptides are in Table 1).

3. Results

The antibodies used as markers for nerves (NFP and S100 protein) exclusively labelled the axons and Schwann cells, in nerve fibers and motor end plates. The antibodies against ASIC2 and TRPV4 immunolabelled both nerves and non-nervous tissues, and both ASIC2 and TRPV4 immunoreactivities were variably found in the oral mucosa, some muscle fibers, and blood (data not shown).

The structure of the cheek consisted of the external skin layer, the superficial musculoaponeurotic system [29,30] containing nerves and blood vessels [18,31], the zygomatic major and buccal muscles [32], and the buccal mucosa. The mean thickness of this region ranged between 1,5 and 2,25 cm (Fig. 1a).

Nerves in the zygomatic major and buccal muscles were regularly observed, both in the connective septa among muscle fascicles and within the muscle itself. The nerve bundles were of different sizes, and run parallel or perpendicular to the direction of the muscle fibers (Fig. 1b and c). The identification putative sensory receptors in the zygomatic major and buccal muscles, was based on the following criteria: independence of the nerves trajectory, be placed in close relation to muscle fibers, and display morphologically differentiated aspect. Moreover, to be regarded as mechanoreceptors immunoreactivity for putative mechanoproteins, must be detected within them. In agreement with these premises we identified capsular corpuscle-like structures of variable size and shape containing numerous axon profiles complexly arranged, which resembled elongated or round Ruffini-like corpuscles (Fig. 2). Although a detailed quantitative analysis was not carried out these structures were regularly found in both muscles (2–4 per section). In addition TRPV4 and ASIC2 immunoreactivity was detected in these corpuscle-like structures, partially co-localized with NFP, this confirming their mechanosensory nature (Fig. 3). In no case were typical muscle spindles found (data not shown), whereas in the masseter muscle (used as a control) typical muscle spindles were found (Fig. S1, Supplementary Material).

4. Discussion

The skeletal muscles contain an intrinsic sensory system, proprioceptive system, which provides information about static and dynamic of joints and muscles [33,34]. Nevertheless, as so far we known no muscle spindles have been found in the human facial muscles [1–4]. In this study, we have not found any morphological or immunohistochemical evidence for the presence of muscle spindles in the two analysed facial muscles, while they were regularly present in the masseter muscle. Therefore, we confirm the absence of typical muscle spindles in human facial muscles.