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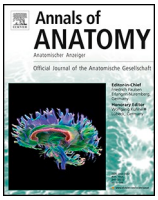

# The human major sublingual gland and its neuropeptidergic and nitrergic innervations

Marianna Boi [a], Roberto Demontis [b], Michela Isola [a], Raffaella Isola [a], Francesco Loy [a], Maria Pina Serra [a], Marcello Trucas [a], Jörgen Ekström [c], Marina Quartu [a,*]

[a] Department of Biomedical Sciences, Section of Cytomorphology, University of Cagliari, Cittadella Universitaria di Monserrato, Monserrato (CA) 09042, Italy
[b] Department of Medical Sciences and Public Health, University of Cagliari, Legal Medicine Division ARNAS Brotzu, Cagliari, Italy
[c] Department of Pharmacology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at University of Gothenburg, Medicinaregatan 13, Box 431, Göteborg 40530, Sweden

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ABSTRACT

*Background:* What textbooks usually call the sublingual gland in humans is in reality a tissue mass of two types of salivary glands, the anteriorly located consisting of a cluster of minor sublingual glands and the posteriorly located major sublingual gland with its outlet via Bartholin's duct. Only recently, the adrenergic and cholinergic innervations of the major sublingual gland was reported, while information regarding the neuropeptidergic and nitrergic innervations is still lacking.

*Methods:* Bioptic and autoptic specimens of the human major sublingual gland were examined by means of immunohistochemistry for the presence of vasoactive intestinal peptide (VIP)-, neuropeptide Y (NPY)-, substance P (SP)-, calcitonin gene related-peptide (CGRP)-, and neuronal nitric oxide synthase (nNOS)-labeled neuronal structures.

*Results:* As to the neuropeptidergic innervation of secretory cells (here in the form of mucous tubular and seromucous cells), the findings showed many VIP-containing nerves, few NPY- and SP-containing nerves and a lack of CGRP-labeled nerves. As to the neuropeptidergic innervation of vessels, the number of VIP-containing nerves was modest, while, of the other neuropeptide-containing nerves under study, only few (SP and CGRP) to very few (NPY) nerves were observed. As to the nitrergic innervation, nNOS-containing nerves were very few close to secretory cells and even absent around vessels.

*Conclusion:* The various innervation patterns may suggest potential transmission mechanisms involved in secretory and vascular responses of the major sublingual gland.

## 1. Introduction

In humans, studies on the innervation pattern of the parenchyma of the three large paired salivary glands have mainly been focused on the parotid and submandibular glands, while less attention has been paid to the major sublingual gland. Recent literature does not always recognize that the so-called sublingual gland in humans is, in reality, a tissue mass consisting of two types of glands (see *e.g.*, Alhajj and Babos, 2023; Ekström et al., 2017; La'Porte et al., 2011; Pedersen et al., 2018; Sreebny and Vissink, 2010), the posteriorly located major sublingual gland, close to the submandibular gland, and the anteriorly located cluster of up to thirty minor sublingual glands (Leppi, 1967; Riva et al., 1988, 1999). The two types of sublingual glands differ in embryonic background (Schulte, 1913) and composition of mucous and seromucous cells (Imai et al., 1982; Riva et al., 1988), as well as in different passages for the saliva to enter the mouth. The major sublingual gland has its outlet by the duct of Bartholin accompanying the submandibular duct, while each of the minor glands has its outlet, i.e., duct of Rivinus, emptying directly into the mouth (Imai et al., 1982; Leppi, 1967; Riva et al., 1988, 1999; Kessler and Bhatt, 2018). Notable, in some laboratory animals, like the

*Abbreviations:* PBS, phosphate-buffered saline; ABC, avidin–biotin–peroxidase complex; CGRP, calcitonin gene-related peptide; nNOS, neuronal nitric oxide synthase; NPY, neuropeptide Y; SP, substance P; VIP, vasoactive intestinal peptide.

* Correspondence to: Department of Biomedical Sciences, Section of Cytomorphology, University of Cagliari, Cittadella Universitaria di Monserrato, Monserrato 09042, Italy.

*E-mail address:* quartu@unica.it (M. Quartu).

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**Table 1**
List of specimens.

| Case | Sex | Age | | |
|---|---|---|---|---|
| **Bioptic specimens** | | | | Pathology |
| 1. | Male | 29 y | | Oral pavement carcinoma |
| 2. | Female | 45 y | | Oral cavity neoplasia |
| 3. | Female | 53 y | | Oral cavity neoplasia |
| 4. | Female | 56 y | | Tongue carcinoma |
| 5. | Male | 59 y | | Tongue leucoplasia |
| 6. | Male | 59 y | | Oral cavity neoplasia |
| 7. | Male | 64 y | | Oral cavity neoplasia |
| 8. | Male | 65 y | | Oral cavity neoplasia |
| 9. | Male | 73 y | | Neoplasia of the trigone |
| **Autoptic specimens** | | | | |
| | | | Post-mortem interval, h | Cause of death |
| 10. | Male | 20 y | 42 h | Myocardial infarction |
| 11. | Male | 44 y | 38 h | Stabbing |
| 12. | Female | 51 y | 45 h | Gunshot wound |
| 13. | Male | 52 y | 52 h | Gunshot wound |
| 14. | Female | 60 y | 48 h | Stabbing |

rat, the anteriorly located minor sublingual glands are separated from the distinct major sublingual gland adhering to the submandibular gland (Greene, 1968; Maynard and Downes, 2019; Redman, 2012).

It was recently realized (Loy et al., 2020) that the innervation of human minor and major sublingual glands do also differ as to their sympathetic adrenergic innervation. Whereas the parenchyma of the minor sublingual gland is virtually devoid of adrenergic nerves, like many other minor salivary glands in humans (Rossoni et al., 1979), adrenergic nerves reach both mucous and seromucous cells as well as secretory ducts in the major sublingual gland (Loy et al., 2020), thus offering an explanation to the sympathetic-induced mucin-rich secretion in response to physical exercise (Ligtenberg et al., 2015). The finding of an adrenergic innervation of the parenchyma of the major sublingual gland agrees with the case in the human parotid and submandibular glands (Garrett, 1967; Norberg et al., 1970). A parasympathetic cholinergic innervation of the parenchyma seems to be an invariable finding in salivary glands (Emmelin, 1967, 1981), and this was also the case in the human major sublingual gland (Loy et al., 2020). In the major sublingual gland, the blood vessels are reached by both cholinergic and adrenergic nerve fibres as in other salivary glands (Garrett, 1967; Norberg et al., 1970).

In addition to cholinergic and adrenergic innervations, there are, to varying extents, also neuropeptidergic and nitrergic innervations of parenchyma and blood vessels in salivary glands, in humans particularly studied in parotid and submandibular glands (Uddman et al., 1980; Hauser-Kronberger et al., 1992; Heym et al., 1994; Matsuda et al., 1997; Kusakabe et al., 1997; Soinila et al., 2006) but also in labial glands (Pedersen et al., 2000). However, no reports appear to be at hand concerning a non-adrenergic, non-cholinergic innervation of the human major sublingual gland.

Therefore, we currently turned our attention to the possible presence of nerve fibres using peptides and nitric oxide (NO) as transmitters, that potentially may affect the functions of the major sublingual gland. Thus, we looked for immunoreactivity to substance P [(SP) an undecapeptide of the tachykinin family and originally shown to cause smooth muscle contraction (Pernow, 1983)], vasoactive intestinal peptide [(VIP), a gut hormone peptide of 28 amino acid residues, named for its long-lasting vasodilatory activity (Said and Mutt, 1970)], neuropeptide Y [(NPY), a pancreatic polypeptide family molecule composed of 36 amino acid residues and initially shown to cause long-lasting vasoconstriction (Lundberg and Tatemoto, 1982)] and calcitonin gene-related peptide [(CGRP), a 37 amino acid peptide acting as potent vasodilator and associated with neurogenic inflammatory responses (Brain et al., 1985; Gamse and Saria, 1985)] as well as for immunoreactivity to neuronal nitric oxide synthase (nNOS), all reflecting transmission mechanisms involved in circulatory, secretory and morpho-functional responses of salivary glands of both animals and humans (Edwards, 1998; Ekström, 1999; Del Fiacco et al., 2015).

**Table 2**
Antibodies used for immunohistochemistry.

| Antigen | Immunogen | Manufacturer, species, and type | Dilution used |
|---|---|---|---|
| **CGRP** | Synthetic rat alpha-CGRP conjugated to bovine serum albumin using glutaraldehyde | CA1134<br>Enzo Life SciencesRabbitPolyclonal | 1:1000 |
| **nNOS** | Recombinant human nitric oxide synthase I (aa 1–200). | AB5380<br>Chemicon®<br>Rabbit polyclonal | 1:1000 |
| **NPY** | Synthetic peptide H-Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-MetAla-Ala-Tyr-Tyr-Ser-Ala-Leu-Arg-Leu-ArgHis-Tyr-Ile-Asn-Leu-Ile-Thr-Arg-Gln-Arg-Tyr-NH2 | T4070<br>BMA Biomedicals, Basel, Switzerland<br>Rabbit polyclonal | 1:500 |
| **SP** | Synthetic peptide conjugated to carrier protein; epitope mapping near the C-terminus of Substance P (RPKPQQFFGLM), an undecapeptide neurotransmitter derived by proteolytic cleavage of protachykinin-1 | sc-21715<br>SantaCruz Biotechnology, CA, USA<br>Rat polyclonal | 1:800 |
| **VIP** | Synthetic peptide corresponding to a sequence in the middle region of human VIP(81–107aa HADGVFTSDFSKLLGOLSAKKYLESLM), different from the related mouse and rat sequences by four amino acids. | RP1108<br>Boster Biological Technology, Pleasanton, CA, USA<br>Rabbit polyclonal | 1:500 |
| **Biotin-conjugated goat anti-rabbit IgG** | Recognizes both heavy and light chains (H+L) | BA-1000, (RRID:AB_2313606) Vector, Burlingame, CA, USA | 1:400 |
| **Biotin-conjugated goat anti-rat IgG** | Recognizes both heavy and light chains (H+L) | BA-9400 (RRID:AB_2336202) Vector, Burlingame, CA, USA | 1:400 |

**Table 3**
Semi-quantitative grading of VIP-, NPY-, SP-, CGRP-, and nNOS-like immunoreactive nerve structures in the human major sublingual gland.

| | Secretory cells | Ducts | Vessels | Nerve bundles |
|---|---|---|---|---|
| **Grade** | | | | |
| 5 many | The lobules involved contained labelled nerve fibres/dot-like terminals covering 75–100 % of the field. | Many ducts were surrounded by labelled filaments and dot-like terminals. | Vessels of different calibre were surrounded by labelled filaments and dot-like terminals. | Stromal nerve fibre bundles of different calibre contained 75–100 % of labelled nerve fibres. |
| 4 modest | The lobules involved contained labelled nerve fibres/dot-like terminals covering 30–75 % of the field. | A number of ducts was surrounded by labelled filaments and dot-like terminals. | A number of vessels of different calibre was surrounded by labelled filaments and dot-like terminals. | Stromal nerve fibre bundles of different calibre contained 30–75 % of labelled nerve fibres. |
| 3 few | The lobules involved contained labelled nerve fibres/dot-like terminals covering 10–30 % of the field. | Occasional ducts reached by isolated labelled filaments and dot-like terminals partially surrounding their profile. | Occasionally, large calibre vessels were reached by isolated labelled filaments and dot-like terminals partially surrounding their profile. | Stromal nerve fibre bundles of different calibre contained 10–30 % of labelled nerve fibres. |
| 2 very few | The lobules involved contained labelled nerve fibres/dot-like terminals covering 5–10 % of the field. | Occasional ducts reached by isolated labelled filaments and dot-like terminals. | Occasional vessels of different calibre were reached by isolated labelled filaments and dot-like terminals. | Occasionally, stromal nerve fibre bundles of different calibre contained a few labelled nerve fibres. |
| 1–0 very few to absent | The lobules involved contained labelled nerve fibres/dot-like terminals covering less than 5 % of the field. | Rare to absent ducts reached by labelled filaments and dot-like terminals. | Rare to absent vascular association with labelled filaments and dot-like terminals. | Stromal nerve fibre bundles of different calibre contained one to none labelled nerve fibre. |

## 2. Material and methods

### 2.1. Sampling

Samples of human major sublingual gland, without clinical alterations, were collected at surgery from nine patients undergoing radical excision for oncological pathologies, who gave their informed consent to participate in the research, and at autopsy from five subjects, with no signs of neuropathology, aged from 20 to 60 years (Table 1). In the case of surgical samples, only tissue fragments, with a size of 2–3 $mm^3$ not compromised by neoplasia or other oral pathologies were selected. Gland eligibility was confirmed by light microscopy evaluation. In compliance with the principles laid out in the Declaration of Helsinki, the study was approved by the Independent Ethics Committee (IEC) of the Azienda Ospedaliero-Universitaria of Cagliari, Italy (Prot. PG/2019/10454 – Part 2.21). The EC has formally stated the moral principles to which the present study adheres. All data were de-identified before database creation and data analysis. Tissues obtained from surgeries were immediately fixed by immersion in phosphate-buffered 4 % paraformaldehyde, pH 7.3, for two hours, rinsed overnight in phosphate-buffered saline (PBS), and paraffin-embedded. Autoptic specimens were fixed by immersion in the same fixative for four hours, rinsed overnight in PBS, and processed either for paraffin embedding or cryostat sectioning.

### 2.2. Immunohistochemistry

Either microtome paraffined sections (5 μm thick) or cryostat sections (14 μm thick), collected on chrome alum-gelatine-coated slides were processed using the avidin-biotin-peroxidase complex (ABC) immunohistochemical technique. The endogenous peroxidase activity was blocked by 0.1 % phenylhydrazine (Cat# 101326606, Sigma Aldrich, St Louis, MO, USA) in PBS) containing 0.2 % Triton X-100 (PBS/T). For the paraffin-embedded tissue, antigen retrieval was then achieved by heating at 90 °C for 20 min in 10 mM citrate buffer (pH 6.0), followed by gradual cooling for 20 min. The sections were then incubated with 20% normal goat serum (S-1000, Vector, Burlingame, CA, USA). Details of the used primary and secondary antibodies are reported in Table 2. The reaction product was revealed by the ABC (Cat#G011–61, BioSpa Div., Milan, Italy), diluted 1:250, followed by incubation with a solution of 0.1 M PB, pH 7.3, containing 0.05 % 3,3'-diaminobenzidine (Sigma Aldrich, St Louis, MO, USA), 0.04 % nickel ammonium sulphate and 0.01 % hydrogen peroxide. All the antisera and the ABC were diluted in PBS/T. Incubation with primary antibodies was carried out for 72 hours at 4 °C. Incubations with secondary antiserum and ABC lasted 60 min and 40 min, respectively, and were performed at room temperature. According to the manufacturers, all used antibodies are immunogen affinity purified. To verify antibody specificity, control immunostainings were run in parallel, either using the same diluted primary antibodies preabsorbed with the respective peptide for 24 h at 4 °C or by omitting the primary antisera, or with PBS/T alone. These procedures resulted in a complete absence of immunostaining in the examined tissues. No liquid phase preabsorption has been carried out for the rat monoclonal antibody against SP, its production and specificity having been previously reported (Cuello et al., 1979) and further characterized in our previous studies (Quartu et al., 1993; Del Fiacco and Quartu, 1994). Slides of some representative specimens were counterstained with either Cresyl Violet or Carazzi's hematoxylin after the immunostaining. Slides were observed using an Olympus BX61 microscope and digital images were captured with a Leica DFC450C camera.

#### 2.2.1. Immunohistochemical evaluation

For a semiquantitative evaluation of VIP-, NPY-, SP-, CGRP-, and nNOS-like immunoreactivities, 200-fold magnification microscopic fields of glandular tissue were examined blindly by a single operator (M. Q.), who was unaware of the origin of specimens, for the presence of nerve fibres and dot-like terminals showing neuropeptide or nNOS labeling associated with secretory cells, ducts, vessels and nerve bundles, by using a five-grade scale, taking inspiration from the grading of neuropeptidergic innervation in a peripheral tissue reported by Witoński and Wągrowska-Danilewicz (2004). In each case, the immunolabeled nerve fibres were scored in five to six microscopic fields of each specimen (see Table 3).

### 2.3. Statistical analysis

One-way ANOVA (followed by multiple comparisons with Tukey's *post-hoc* test) was used to analyse the semiquantitative arbitrary values scored for the samples of different origin and age and immunolabeled for the VIP, NPY, or SP. Pearson's test was used for the correlations. The statistical analyses were performed with PRISM, GraphPad 6 Software (San Diego, CA, USA).

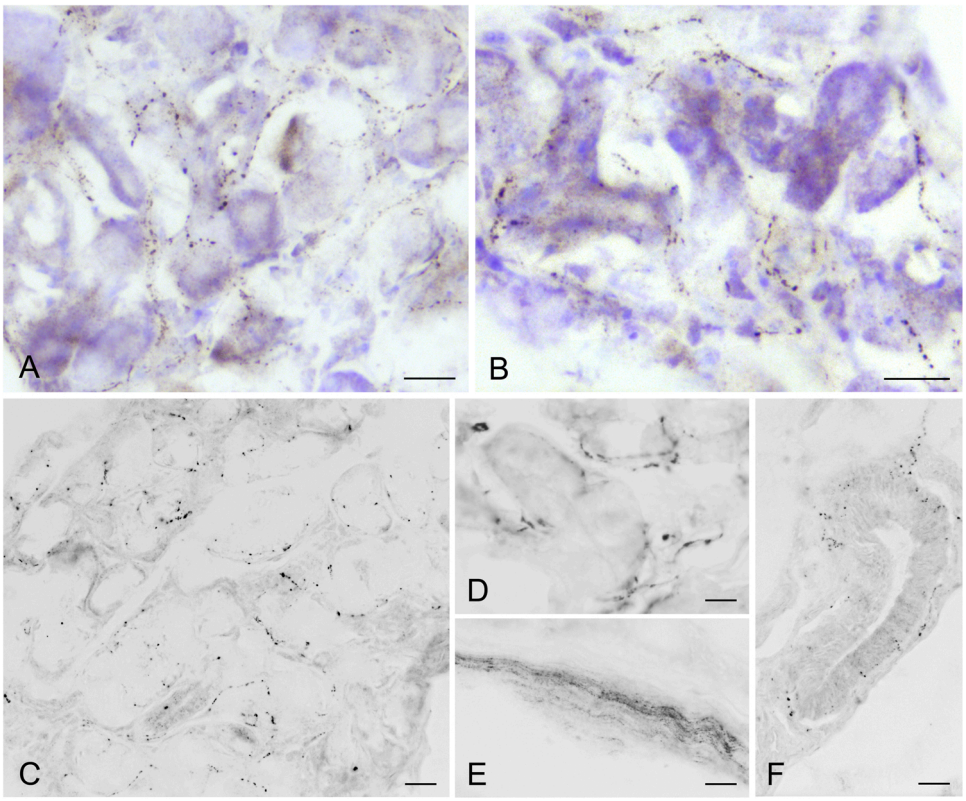


**Fig. 1.** Vasoactive intestinal polypeptide (VIP)-like immunoreactive nerve fibres in the human major sublingual gland, counterstained with cresyl violet in A, B. Tracts of immunostained varicose nerve fibres occur in close proximity to secretory cells (A-D), intercalated (B) and striated ducts (F), and in nerve bundlets (E). A, B: case 13; C, E, F: case 12; D: case 9. Scale bars: A, B, C, E, F = 50μm; D = 25μm.

## 3. Results

All the examined samples of the major sublingual glands contained structures immunoreactive to VIP (Fig. 1), NPY (Fig. 2 A-C), SP (Fig. 2 D-F), CGRP (Fig. 2 G), and nNOS (Fig. 2 H). Apart from the size of the specimens, being tiny as bioptic tissue fragments to large as autoptic tissue specimens, no patent morphological differences were appreciable among the examined specimens. A representative control VIP-immunostaining in shown in Fig. 3. Neuropeptide-immunostained structures were mostly represented by tracts of nerve fibres, with a varicose aspect, running near mucous tubular cells, seromucous cells, ducts, blood vessels, and by immunostained nerve fibre bundles running in the connective tissue (Fig. 1 D; 2 F, I). In VIP-immunostained sections (Fig. 1), labelled thin varicose nerve fibres and dot-like terminals of different calibre, from tiny to coarse, were observed adjacent to the basal surface of secretory cells or running in the connective tissue surrounding the ductal epithelial cells. Very few NPY- (Fig. 2 A-C) and SP-labelled (Fig. 2 D-F), and rare to none nNOS-immunostained nerve fibres were observed in proximity of secretory tubular cells (Fig. 2 H), while only SP-like immunoreactivity labelled rare nerve fibres approaching the ductal epithelial cells. In the VIP- and SP-immunolabeled sections of some specimens examined, occasional neuronal perikarya localized in the loose connective tissue between the secretory lobules could be observed (Fig. 4). Morphological distribution and semiquantitative evaluation of immunohistochemical labeling of nerve fibres associated with the parenchymal and stromal structures of the human major sublingual gland are summarized in Table 4.

Because of their constant occurrence in the secretory lobules compared to the other markers, the semiquantitative arbitrary values scored for the VIP-, NPY-, and SP-like immunoreactive nerve structures allowed the statistical evaluation. The one-way ANOVA showed no statistical differences among the immunoreactivities of the different examined specimens for each marker [for VIP, $F(9, 45) = 2.017$; for NPY, $F(9, 29) = 1.622$; for SP, $F(9, 33) = 1.651$]. The Pearson's correlation test showed that, for each examined neuropeptide-labelling, the semiquantitative mean arbitrary values did not differ by gender ($r = 0.325$) nor by source of sampling (biopsy vs autopsy) ($r = 0.368$).

## 4. Discussion

The innervation of parenchyma and vessels of the human major sublingual gland seems to be an unexplored field of research except for a recent study explicitly focusing on the gland and its cholinergic and adrenergic innervation (Loy et al., 2020). The present study provides further information by investigating the gland's neuropeptidergic and nitrergic innervation.

Several available literature data give evidence for the immunodetectability and determination of various neuropeptides and trophic proteins in neuronal structures of the human autoptic nervous tissue (Quartu et al., 1992; 1993; Del Fiacco and Quartu, 1994; Serra et al., 2005; Del Fiacco et al., 2014), and neuropeptides and enzymes in salivary gland innervation (Loy et al., 2020; Sato et al., 2022). In this context, it is worth mentioning that all samples of major sublingual glands showed similar localization and distribution of specific immunolabeling regardless of their bioptic or autoptic source.

The human submandibular ganglion is usually thought to provide the sublingual gland with postganglionic parasympathetic nerves. This ganglion of (foetal and adult) humans shows immunoreactivity to VIP and nNOS (Kiyokawa et al., 2012; Kawashima et al., 2021). However, recent human cadaver dissections have drawn attention to the presence of a sublingual ganglion, located on the side of the sublingual gland, that, in addition to the submandibular ganglion, provides the sublingual gland with postganglionic parasympathetic nerves (Takezawa and Kageyama, 2015; Iwanaga et al., 2023); a sublingual ganglion, that

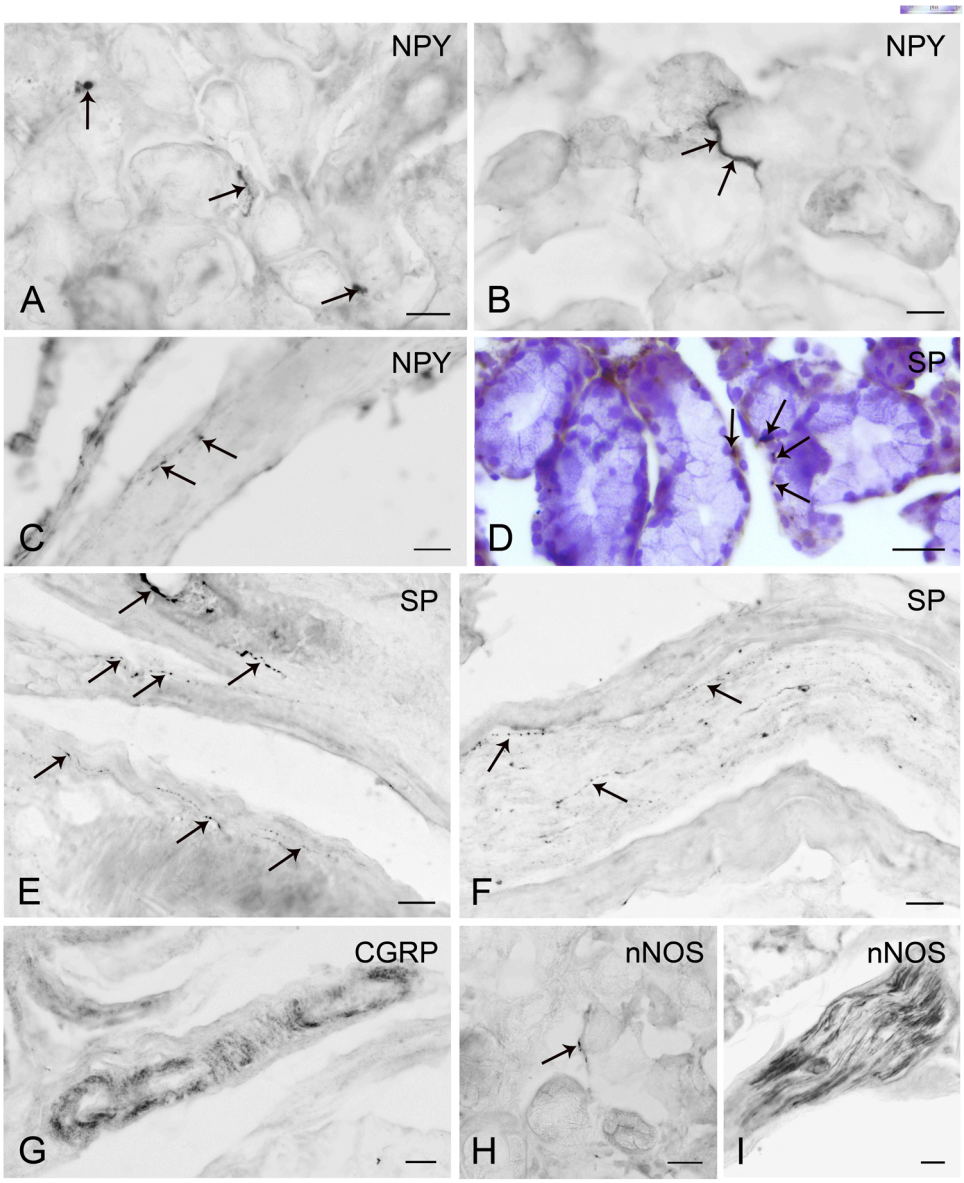


**Fig. 2.** Neuropeptide Y (NPY)- (A-C), substance P (SP)- (D-F), calcitonin gene-related peptide (CGRP)-(G), and neuronal nitric oxide synthase (nNOS)-like immunoreactive (H, I) nerve fibres in the human major sublingual gland. Tracts of immunostained fibres (A, B, H) and coarse dot-like terminals (D, with Carazzi's hematoxylin counterstaining) occur near secretory cells, vessels (C, E, G), and in nerve bundlets (F, I). A, E, H, I: case 14; B: case 2; C: case 10; D, G: case 13; F: case 12. NPY: neuropeptide Y; SP: substance P; CGRP: calcitonin gene-related peptide; nNOS: neuronal nitric oxide synthase. Scale bars: A, D, E-G, I = 50μm; B, C = 25μm; H = 10 μm.

seems to have fallen into oblivion (Ozyurt, 2022). The present observation of occasionally occurring interlobular cell bodies displaying SP- and VIP-immunolabeling serves to underline the existence of points of relay distal to the submandibular ganglion.

In the connective tissue of the major sublingual gland, bundles of nerves showing immunoreactivity to the neuropeptides under study as well as to nNOS were observed. Of the various nerve populations under study, those showing immunoreactivity to VIP were the most abundant. VIP-containing nerves were found close to secretory cells, ducts, and blood vessels, a distribution pattern similar to that in human parotid and submandibular glands (Uddman et al., 1980; Hauser-Kronberger et al., 1992; Heym et al., 1994; Matsuda et al., 1997; Kusakabe et al., 1997; Garrett, 1999; Del Fiacco et al., 2015). VIP is known to be co-localized with acetylcholine (Lundberg et al., 1988; Lundberg, 1996; Kawashima et al., 2021), and parasympathetic denervation causes the VIP-containing nerves as well as the gland content of VIP almost to disappear, as shown in parotid glands of experimental animals (Ekström et al., 1984; Tobin et al., 1990; Khosravani et al., 2008). In the human major sublingual gland, the NPY-positive innervation was poor; only a few NPY-containing nerves were found to reach secretory cells and vessels. In contrast, the human parotid and submandibular glands display a rich NPY innervation of secretory cells, ducts, and vessels (Matsuda et al., 1997; Hauser-Kronberger et al., 1992; Kusakabe et al., 1997). In human parotid and submandibular glands, NPY-containing nerves innervating the vessels show, in addition, tyrosine hydroxylase immunoreactivity, indicating that these nerves are of sympathetic origin (Heym et al., 1994). In line with this observation is the outcome of denervation experiments of the rat parotid gland, showing NPY-containing nerves around ducts and blood vessels to be of sympathetic origin, while those NPY-containing nerves around secretory cells (and also containing VIP) to be of parasympathetic origin (Schultz et al., 1994; Ekström et al., 1996). In the human major sublingual gland, few to very few SP-containing nerves were occasionally found close to secretory cells, ducts, and vessels. Also in the two other major glands,

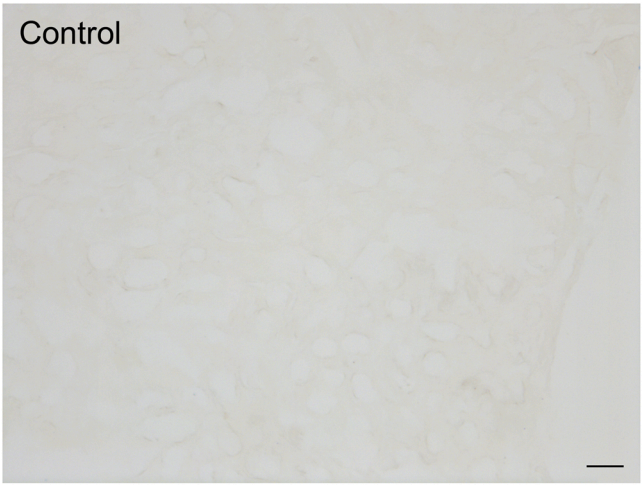


**Fig. 3.** Control immunostaining of a section of the human major sublingual gland achieved using the primary antibody against the vasoactive intestinal polypeptide (VIP) preabsorbed with the relevant peptide antigen. Case 12. Scale bar: 50μm.

SP-containing nerves close to secretory cells and ducts are scarce or even lacking, while some SP-containing nerves usually are reported around vessels (Hauser-Kronberger et al., 1992; Kusakabe et al., 1997; Matsuda et al., 1997; Del Fiacco et al., 2015). SP and VIP have been shown to co-exist in periacinal cholinergic nerves of salivary glands (Al-Hadithi et al., 1988; Garrett, 1999). In the major sublingual gland, CGRP-containing nerves were only observed sporadically and, if so, close to blood vessels. In human parotid and submandibular glands, the picture seems similar to secretory cells and ducts, while a small number of CGRP-containing nerves occur regularly around vessels (Hauser-Kronberger et al., 1992; Kusakabe et al., 1997; Matsuda et al., 1997). In the human parotid gland, a co-localization between SP and CGRP was reported in some nerves close to ducts and frequently around vessels (Heym et al., 1994). Nerves containing SP/CGRP are thought to be of sensory origin (Saria et al., 1985; Sundler et al., 1985), and in rat parotid gland these nerves disappear in response to the sensory neurotoxin capsaicin (Ekström et al., 1989). A possible SP/CGRP co-localization was not looked for in the current study. In the rat parotid gland, parasympathetic denervation showed almost all SP-containing nerves (lacking CGRP) to disappear, whereas CGRP nerves (most of which contained SP) seemed unaffected (Ekström et al., 1989). Though labelled bundles of intensely positive nerves for nNOS were observed in the human major sublingual gland, single fibres innervating secretory elements or vessels were hard to observe. Although not clearly expressed whether the major sublingual gland was examined, Soinila et al. (2006) reported that the sublingual gland, like the parotid gland, only occasionally displayed nNOS-containing nerves, and Asakawa et al. (2015) only found interlobular nerves to express nNOS. Animal experiments have shown nNOS terminals to encircle acini, ducts, and vessels and further, that these nerves are of parasympathetic origin (Alm et al., 1995; 1997).

Reports on neuropeptidergic and nitrergic innervations on the human *minor sublingual* glands appear to be lacking. However, some other types of minor salivary glands have been studied, *i.e.*, particularly the labial gland but the findings vary e.g., as to acini and ducts from rare to abundant concerning VIP, and absent and few, respectively, with NPY, while both neuropeptides were found close to vessels but also here in varying quantity (Sato et al., 2022; Pedersen et al., 2000). A scarce SP-innervation of acini, ducts, and vessels was found by Pedersen et al. (2000). Sato et al. (2022) also examined palate, lingual, and Ebner's glands. Whereas the VIP-innervation of the acini of the palatal and lingual glands, both with predominantly mucous cells, was numerous, the NPY-innervation of the acini was scanty in Ebner's gland, consisting of mainly serous cells like the labial glands (Sato et al., 2022). In labial glands, a (very) sparse innervation of nNOS of acini, ducts, and vessels has been reported (Konttinen et al., 1997; Pedersen et al., 2000; Soinila et al., 2006).

In analogy with known effects of various transmitter actions on target cells in salivary glands predominantly studied in animals (Lundberg et al., 1988; Edwards, 1998; Ekström, 1999; Del Fiacco et al., 2015), potential complementary actions, albeit to various degrees, might be expected in the human major sublingual gland, based on its immunoreactivity to neuropeptides and neuronal nitric oxide synthase e.g., VIP as to protein (and fluid) secretion and vasodilatation; NPY as to protein (and fluid) secretion and vasoconstriction; SP as to vasodilation (and fluid secretion); CGRP as to vasodilation (and protein secretion); and possible nNOS generated NO as to secretion. It should particularly be mentioned that SP is known as a very potent secretagogue in some

**Table 4**
Distribution and semiquantitative evaluation of the neuropeptide-and the nNOS-like immunoreactive nerve fibres in the human major sublingual gland.

| | Secretory cells | Ducts | Vessels | Nerve bundles |
|---|---|---|---|---|
| VIP | 5 | 2 | 4 | 4 |
| NPY | 2 | 0 | 2 | 3 |
| SP | 2 | 2 | 3 | 4 |
| CGRP | 0 | 0 | 3 | 4 |
| nNOS | 1–0 | 1–0 | 0 | 4 |

Legend: 5 many, 4 modest, 3 few, 2 very few, 1–0 very few to absent.

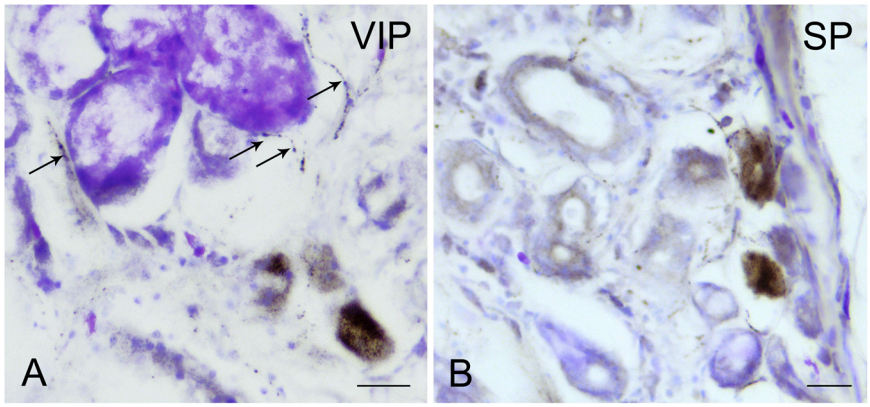


**Fig. 4.** Vasoactive intestinal polypeptide (VIP)- (A) and Substance P (SP)-like immunoreactive neuronal cell bodies (B) within the human major sublingual gland counterstained with cresyl violet. Arrows in (A) point to VIP-like immunoreactive varicose nerve fibres. Case 12. VIP: vasoactive intestinal peptide; SP: substance P. Scale bars: A, B = 50μm.

**Table 5**
Summary of literature regarding the neuropeptidergic innervation of the major sublingual gland of various species.

| | | | | VIP | SP | NPY | CGRP | Galanin | ENK | Bombesin |
|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | **References** | **Method** | **Intraglandular structures** | | | | | | | |
| Cat | - Wharton et al. (1997)*<br>- Uddman et al. (1980)*<br>- Lundberg et al. (1988)# | # IHC<br>* RIA ± IHC | Secretory cells<br>Ducts<br>Vessels | Yes<br>Yes<br>Yes | No<br>No<br>Yes | | No<br>No<br>Yes | | | |
| Ferret | - Tobin et al. (1990)#<br>- Khosravani et al. (2008)* | # IHC<br>* RIA ± IHC | Secretory cells<br>Ducts<br>Vessels | Yes<br>Yes<br>Yes | Yes<br>Yes<br>Yes | Yes<br>Yes<br>Yes | Yes<br>Yes<br>Yes | Yes<br>Yes<br>Yes | | |
| Guinea-pig | - Gibbins (1990)#<br>- Morris et al. (1997)# | # IHC | Secretory cells<br>Ducts<br>Vessels | Yes<br>Yes<br>Yes | Yes | Yes<br>Yes<br>Yes | Yes | | Yes<br>Yes<br>Yes | |
| Rat | - Wharton et al. (1997)#<br>- Ekström et al. (1984)*<br>- Ekström et al. (1988)*<br>- Soinila et al. (1991)#<br>- Konopka et al. (1992)*<br>- Schultz et al. (1994)#<br>- Aalto et al. (1997)* | # IHC<br>* RIA ± IHC | Secretory cells<br>Ducts<br>Vessels | Yes<br>Yes<br>Yes | Yes<br>Yes<br>Yes | Yes<br>Yes<br>Yes | Yes<br>Yes<br>Yes | Yes<br>Yes | Yes<br>Yes<br>Yes | Yes |
| Sheep | - Wathuta (1986)# | # IHC | Secretory cells<br>Ducts<br>Vessels | Yes<br>Yes<br>Yes | No<br>No<br>No | | | | | No<br>No<br>No |

Legend: IHC, immunohistochemistry; ENK, enkephalin; NPY, neuropeptide Y; RIA, radioimmunoassay; SP, substance P; VIP, vasoactive intestinal peptide

species but not in humans, as judged by observations on parotid and submandibular glands (Larsson et al., 1986; Del Fiacco et al., 2015). Possible positive interactions with focus on the secretion (fluid or protein secretion or both) might be expected in the major sublingual gland in response to VIP combined either with SP or the classical transmitters acetylcholine or noradrenaline (on its action on $\alpha_1$-adrenergic receptors) and to CGRP combined either with SP or acetylcholine and further, with focus on vasodilation to VIP combined with acetylcholine and to CGRP combined with SP (Lundberg, 1996; Edwards, 1998; Ekström, 1999). Though the uncertain contribution of NO in functional responses of the major sublingual gland, as judged from the current morphological findings, it may be mentioned that animal experiments show the effect of VIP to be in part NO-dependent not only as to protein secretion and vasodilation, following its release from the nerve endings, but also on its release from the nerve terminals due to NO acting presynaptically (Edwards, 1998). Parasympathetic ganglia, including the otic and submandibular ganglion, display co-localization of NOS and VIP, in both animals and humans (Ceccatelli et al., 1994; Alm et al., 1995; Uddman et al., 1999).

The current study on the human major sublingual gland focused on neuropeptides that have been relatively extensively studied in salivary glands as to their origin, presence, release, and function (Lundberg et al., 1988; Edwards, 1998; Garrett, 1999; Ekström, 1999; Khosravani et al., 2008; Del Fiacco et al., 2010; 2015). The possible contribution of some further neuropeptides to the innervation of the human major sublingual gland should be recognized judging from previous findings in salivary glands of both humans and animals. Of particular interest, seem to be the neuropeptides bombesin (Aalto et al., 1997), galanin (see e.g. Konopka et al., 1992; Kusakabe et al., 1997; Del Fiacco et al., 2010), somatostatin (see e.g. Matsuda et al., 1997; Ostuni et al., 1999), pituitary adenylate cyclase-activating peptide, PACAP, (see e.g. Tobin et al., 1995; Mirfendereski et al., 1997), the VIP-like peptide histidine methionine, PHM, (see e.g. Hauser-Kronberger et al., 1992; Del Fiacco et al., 2015), and enkephalin (see e.g. Gibbins, 1990; Franzén et al., 1993). Table 5 summarizes reports on the presence of neuropeptides particularly in the major sublingual gland of some animal species, demonstrated by visualizing the peptidergic nerves and/or measuring the neuropeptide gland content. The presence of a VIP-ergic innervation seems to be a regular finding, while the SP-ergic and CGRP-ergic innervations vary between the species. So far, NPY-ergic innervation seems to be a regular phenomenon among the few species examined. Some studies have included observations on the presence of galanin, enkephalin, or bombesin.

The present data are intended to contribute to the knowledge of peptidergic and nitrergic innervation in the human major sublingual gland in normal conditions. The study of human tissue in normal conditions represents a mandatory step into the possibility of translating experimental and preclinical studies to the clinic. Certainly, the study of human tissue is partly hampered by the limited availability of specimens and, markedly in the case of the major sublingual gland, by the intrinsic heterogeneity of available samples for age, size, and the ratio between secretory and stromal components.

Eventually, it should be pointed out that the anatomy of the Bartholin duct draining the major sublingual gland varies. The duct may open directly, separated from the submandibular duct, at the sublingual caruncle, or join the submandibular duct to form a common draining orifice (Zhang et al., 2010). Thus, in humans, it is particularly difficult to avoid contamination of the saliva secreted from the major sublingual by submandibular saliva. In both cases, the saliva collected by the devices in use will obtain a mixture of saliva from the two glands (Sreebny and Vissink, 2010). Thus, *in vitro* studies of surgical specimens of the major sublingual gland seem to offer advantages allowing for exposure of gland tissues to various agonists followed by analyses of constituencies in the medium as well as of ultrastructural phenomena induced by the agonists (Cabras et al., 2008; Loy et al., 2015; Del Fiacco et al., 2015).

## 5. Conclusion

To conclude (and with a focus on secretory cells and vessels), the major sublingual gland displayed, compared with the two other major glands, on the whole, a similar rich VIP-ergic innervation, a poorer NPY-ergic innervation and a similar poor SP- and CGRP-containing innervation. Like in the two other types of major glands, the nNOS-labeled innervation was poor in the major sublingual gland. The various transmission mechanisms, suggested by the current immunohistochemistry, are likely to play roles in secretion and blood flow of the gland.

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## Ethical approval details

The study was approved by the Independent Ethics Committee (IEC) of the Azienda Ospedaliero-Universitaria of Cagliari, Italy (Prot. PG/2019/10454 – Part 2.1).

## CRediT authorship contribution statement

**Marianna Boi:** Methodology, Investigation, Data curation. **Roberto Demontis:** Methodology, Data curation. **Michela Isola:** Data curation. **Raffaella Isola:** Data curation. **Francesco Loy:** Resources, Data curation. **Maria Pina Serra:** Writing – review & editing, Resources, Methodology, Funding acquisition, Data curation. **Marcello Trucas:** Data curation. **Jörgen Ekström:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Investigation, Funding acquisition, Conceptualization. **Marina Quartu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of Competing Interest

None.

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