

A QUANTITATIVE STUDY OF NERVE FIBER DENSITY IN THE SUBMANDIBULAR GLAND OF RATS

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ABSTRACT

The route and three dimensional distribution of nerve terminals in the submandibular gland were investigated in rats using immunohistochemistry for the protein gene product (PGP) 9.5, as a marker of neuronal elements.

Thick fiber bundles were found along the wall of the excretory duct. Many fine fibers from these thick bundles were distributed each lobule of the submandibular gland. A large number of single fibers terminated in the area around the striated, intercalated ducts and the acini. The densities of PGP 9.5 immunoreactive terminals were measured by a computer aided analysis system in the three areas: the striated duct, the intercalated duct, and the acini, whose densities ($\mu\text{m}/\mu\text{m}^2$) were 0.23, 0.39 and 0.05 respectively.

The relatively high density of nerve terminals in the intercalated duct suggests that the duct system probably plays an unexpectedly important role in the functional aspects.

Key Words: Salivary gland, Nerve distribution, PGP 9.5, Avidine and biotin-peroxidase complex, Rats

INTRODUCTION

In the submandibular glands, the lobular parenchyma contains many terminal secretory endopieces consisting of the acini and the duct system, i.e., the intercalated, the granular and the striated ducts. Current physiological studies have provided evidence that the epithelium of each area comprising the terminal secretory endopiece exerts some characteristic influence on salivation. The epithelial cells of the acinus mainly produce saliva, and the ductal epithelium functions as one of the composers producing saliva by controlling the osmotic pressure of secreted fluid.¹⁾ On the other hand, an early neuroanatomical study using the silver impregnation method has shown that various kinds of peripheral nerves, including the parasympathetic, sympathetic and sensory nerves, were innervated into the rat submandibular gland.²⁾ Current immunohistochemical studies have also reported that different kinds of nerves were more precisely innervated in the submandibular gland.^{1,3,4)} Although these early studies revealed that

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well-developed nerve fibers played an important role in salivation in the submandibular gland, the relation between the regional variations in epithelial function and the innervation patterns in each area remains unclear due to a dearth of two-dimensional data on the regional variations in nerve distribution in each area of the secretory endpiece. Although the silver impregnation method is usually performed to demonstrate the complicated nervous networks in tissues, it has proven to be unsuitable for investigating the thick sections that comprise part of the total structure of the secretory endpiece. To clarify the three-dimensional structure of nerve fibers in tissues, we must overcome the technical problems facing current histological procedures. Our interest, therefore, must be focused on the three-dimensional relation between nerve terminals and epithelial structures at each area of the secretory endpiece.

The neuron-specific protein gene product 9.5 (PGP 9.5) is a cytosolic 24.8 kDa protein that was originally discovered by high-resolution two-dimensional mapping of soluble proteins from the human brain.⁵⁾ Early immunohistochemical studies have indicated that this protein is widely distributed and highly expressed in vertebrate neurons and neuroendocrine cells in general organs.⁶⁻⁹⁾ This protein as well as the neuron-specific enolase is now used as an excellent marker for the neuronal components. Nerve growth factor (NGF) is a 26-kDa hemodimer of 13-kDa polypeptides, referred to as β NGF.¹⁰⁾ During developmental stages, the axons from sympathetic neurons are induced by a high concentration of NGF.^{11,12)} In adult animals, NGF also acts as a neurotrophic factor on some neuronal populations, including the sympathetic and sensory neurons and the basal forebrain cholinergic neurons.¹³⁻¹⁵⁾

The present study was designed to express the three-dimensional construction and density of nerves in each area of the submandibular gland from the main duct to the acini, especially in the region surrounding a secretory endpiece. In addition, we speculated on the correlation between NGF-positive epithelium and the areas with dense innervation.

MATERIALS AND METHODS

Animals and tissue preparation

All experimental procedures in this study conform to the animal experimentation guidelines of the Nagoya University Graduate School and Faculty of Medicine.

Male Wistar rats (n=5), 11-weeks-old and around 350g body weight, were anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg), and perfused with 350 ml of saline followed by the same volume of 4% paraformaldehyde in 0.1 M phosphate buffer. After perfusion, the bilateral submandibular glands were dissected out and immersed in the same fixative overnight. Under an operating microscope, the surrounding connective tissues were removed from the main secretory duct and the body of the submandibular gland in phosphate buffered saline (PBS). The body of the submandibular gland was cut in two, one half of which was embedded in 4% gelatin and cut on a vibrating microtome (Vibratome, Technical Products International, St. Louis, USA) into 200 μ m sections that were then collected in a multi-well plate filled with ice-cold PBS. The other half was immersed in a 20% sucrose solution overnight and then rapidly frozen at -80° . Serial 15 μ m sections were cut on a cryostat and mounted on a glass slide coated with chrome alum gelatin. The entire of the secretory duct tissues and thick vibratome sections were dehydrated in an ascending series of ethanol and delipidated with xylene until they became clear. The tissues were then rehydrated in a descending series of ethanol to PBS, and kept in ice-cold PBS until immunoreaction. The entire of the secretory duct and thick vibratome sections were used for PGP 9.5 immunostaining, and the cryostat sections for NGF immunostaining.

Immunohistochemistry

1) PGP 9.5 immunostaining

Free-floating vibratome sections and the entire of the secretory duct tissues were incubated in PBS containing anti-PGP 9.5 antisera (UltraClone Limited, England, UK) at a dilution of 1:5000 with PBS containing 0.3% Triton-X 100 for 4 days at 4°C. Samples were rinsed with PBS and reacted with biotinylated anti-rabbit IgG (VECTOR, U.K.) diluted 1:200 in PBS for 2 hours, followed by a solution of avidin and biotin-peroxidase complex at a dilution of 1:100 in PBS for 90 minutes. Antibody-binding sites were made visible with a peroxidase-DAB reaction. All procedures except for step of the primary antibody step were performed at room temperature.

2) NGF immunostaining

Anti-NGF rabbit serum (Santa Cruz, CA, USA) was diluted at a concentration of 1:1000 with PBS containing 0.3% Triton-X 100. About 100 μ l of anti-sera solution was dropped on each section. Sections were incubated at 4°C for 4 days, and then rinsed with PBS and reacted with PBS containing biotinylated anti-rabbit IgG (VECTOR, U.K.) at a dilution of 1:200 with PBS for 2 hours, followed by a solution of avidin and biotinylated peroxidase complex at a dilution of 1:100 (ABC, VECTOR, U.K.) at a concentration of 1:100 in PBS for 90 minutes. After rinsing with PBS, sections were made visible with a peroxidase-DAB reaction. All procedures except for immuno-reaction with the primary antibody were performed at room temperature.

The purpose of this study was to clarify the differences in nerve innervation among portions of the submandibular gland, which is a branched tubuloacinar gland with a terminal secretory portion consisting of striated and intercalated ducts, and serous or mucous acini. Under light microscopy, sizes of the whole body of the terminal secretory portion were estimated to range from 100 to 150 μ m. Thus, we used 200 μ m thick sections for the immunohistochemical observation of nerve fibers distributed in the submandibular gland.

After sufficient infiltration of antibodies to the thick vibratome sections, the sections were immersed in xylene. Triton X-100, as a detergent, was mixed with the incubation medium to dilute the antibodies, and endogenous peroxidases were blocked by a solution of methyl-alcohol including 0.3% H₂O₂. In addition, sections were placed in a low-concentration solution of antibodies for a long period at low temperature. These procedures contributed to achieving staining results sufficient to demonstrate a complete image of nerve endings distributed around the terminal secretory portion of the submandibular gland. As a control for the immunostain, the primary antibody was replaced by non-immune rabbit IgG in the same dilution as the primary antibody. No unspecific immunostain was observed.

3) Data collection

Post-stained tissues were examined under a light microscope (Nikon, Tokyo, Japan) equipped with a computer-aided, three-dimensional reconstructive system (NeuroLucida, Micro Bright Field Inc., Colchester, VT, USA). The histological structure of the terminal secretory portion was re-identified by comparing it with Hematoxylin-Eosin stained sections.

The numerical values were collected from 50 points in each section with a notable acinus-duct structure. The total length of immunoreactive fibers was measured in the area around the striated duct, the intercalated duct including the granular duct, and the acinus. The density of nerve fibers was expressed as the value arrived at by dividing the total length of fibers by the area of each part.

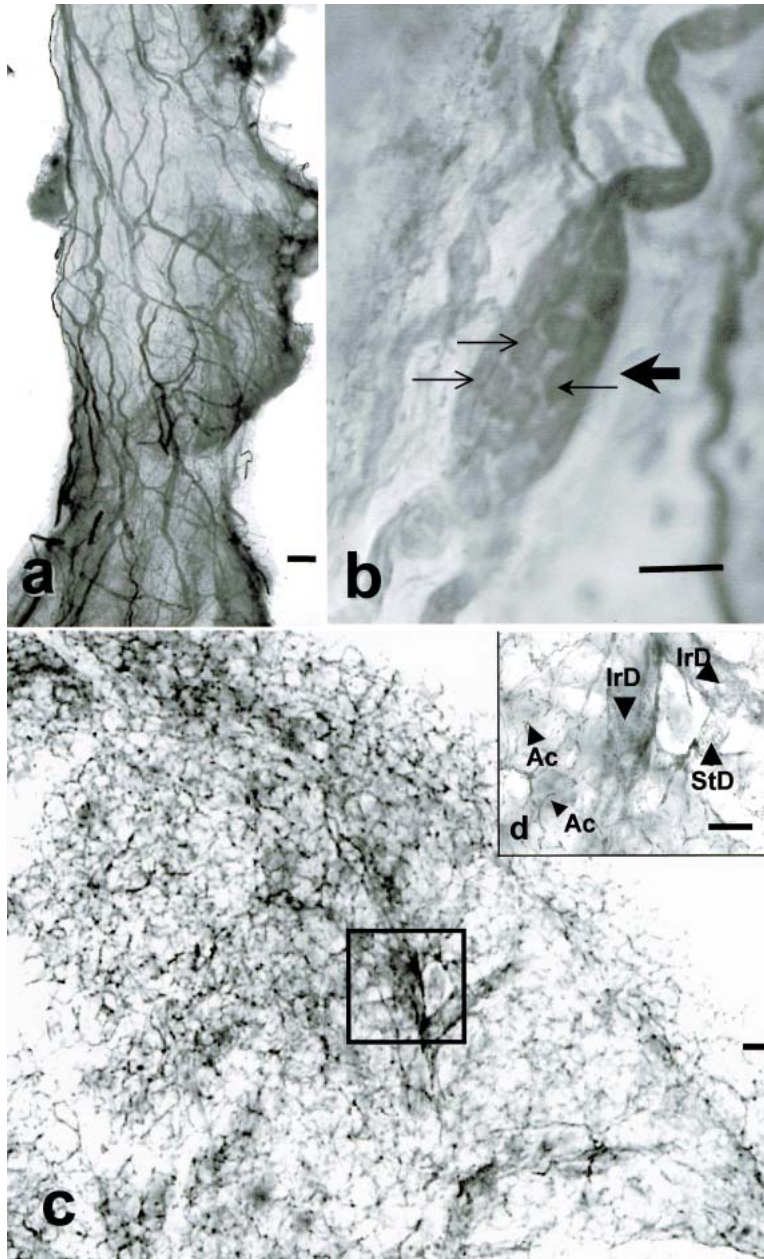


Fig. 1 Light microscopic feature of part of the submandibular duct (a). Many fiber bundles formed complicated networks on a wall of the submandibular duct. There were many nerve bundles containing PGP 9.5 immunoreactive fibers. Periductal ganglia were sometimes found on the bundles (large arrow in b), and contained many ganglion cells (small arrows in b).
 c: Light-micrographs of part of the submandibular gland at low magnification of PGP 9.5 staining. The innervation in a lobule of the rat submandibular gland was seen.
 d: High magnification of Fig. 1-c. Fine nerve fibers have branched out from the fiber bundles on the intralobular duct (IrD), have entered into the striated duct (StD), and have then distributed around the acini (indicated by "Ac"). Scale Bar = 50 μ m.

RESULTS

Histochemical findings

After immunostaining, PGP 9.5 immunoreactive fibers were recognized by a dark-brownish deposition of peroxidase-DAB reaction products, clearly distinguishing them from immunonegative structures. Fiber bundles ramified from thick fiber bundles sometimes branched out into several small bundles, and eventually forming a reticular structure around the duct (Fig. 1-a). Periductal ganglia were sometimes found on the bundles (large arrow in Fig. 1-b), and seen to contain many ganglion cells (small arrows in Fig.1-b).

Many fine fiber bundles branched out from these small bundles, entered into the parenchyma along the intralobular duct (IrD) (Fig. 1-c), and were distributed in an area around the intralobular duct (Fig.1-c). In the periphery of the parenchyma, the fibers branched out to several single fibers that terminated in the area around the striated and intercalated ducts and the acini (Fig. 1-d). Upon PGP 9.5 immunostaining of these fibers, we could not distinguish the granular from the intercalated ducts, so that the granular duct was shown to be involved in the intercalated duct areas.

NGF immunoreactivity was found in the epithelial cells of the granulated and striated ducts, but was not dominant in the epithelial cells of the acini (Fig. 2).

Fiber density

Camera Lucida drawings of nerve bundles containing PGP 9.5 immunoreactive fibers on the rat submandibular gland are shown (Fig. 3-a,b). The submandibular duct is divided into the interlobular ducts (IID) at the hilus. Periductal ganglia (PDG) are found on the bundles (Fig. 3-a).

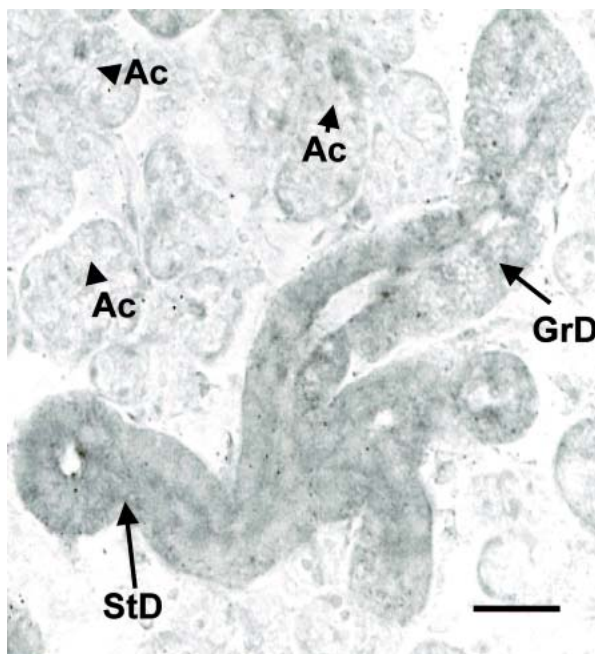


Fig. 2 Light-micrograph of NGF-immunoreactive epithelial cells located in granulated duct (GrD), striated duct (StD), and all over the lobule (Ac: acinus).

Fig. 3-b depicts an overview of the PGP 9.5 immunoreactive fibers distributed in a lobule of the submandibular gland. The arrow indicates the direction from which the nerve fibers enter this lobule. Fig. 4-a, -b and -c shows three-dimensional views of PGP 9.5 immunoreactive fibers are observed around the terminal secretory portion. Rotated views (Fig. 4-a,b) clearly demonstrate the terminal acinus branching of nerve fibers under different aspects, showing an orderly branching of small bundles into single fibers from the interlobular duct to the terminal acinus. Double black arrows indicate single fiber on each terminal acinus after the rotation (Fig. 4-b).

Under the microscope, the area of the terminal secretory portion was identified and color coded (Fig. 4-c).

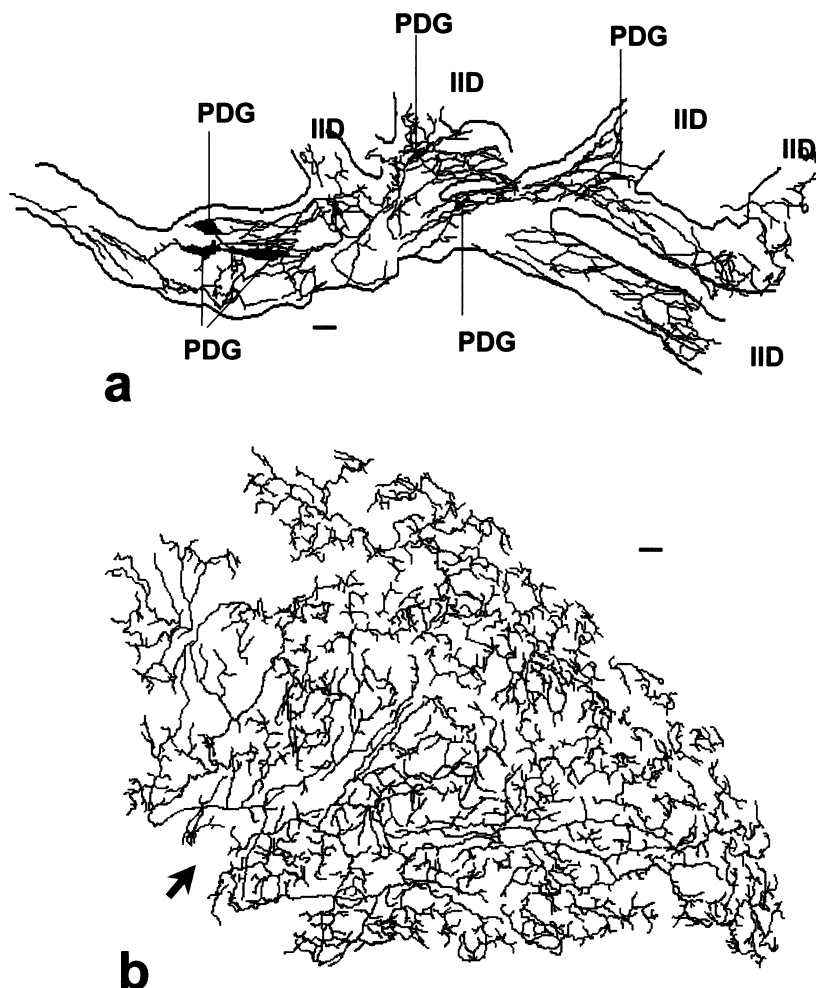


Fig. 3 Camera Lucida drawings of nerve bundles containing PGP 9.5 immunoreactive fibers on the rat submandibular gland (a and b). Submandibular duct is divided into five interlobular ducts (IID) at the hilus of submandibular gland. Many fiber bundles were seen on the wall of submandibular duct (a). Five periductal ganglia (PDG) were found on the bundles. Overview of PGP 9.5 immunoreactive fibers distributed in a lobule of submandibular gland (b) Arrow indicates direction from which the nerve fibers enter this lobule.

NERVE DISTRIBUTION IN RAT SALIVARY GLAND

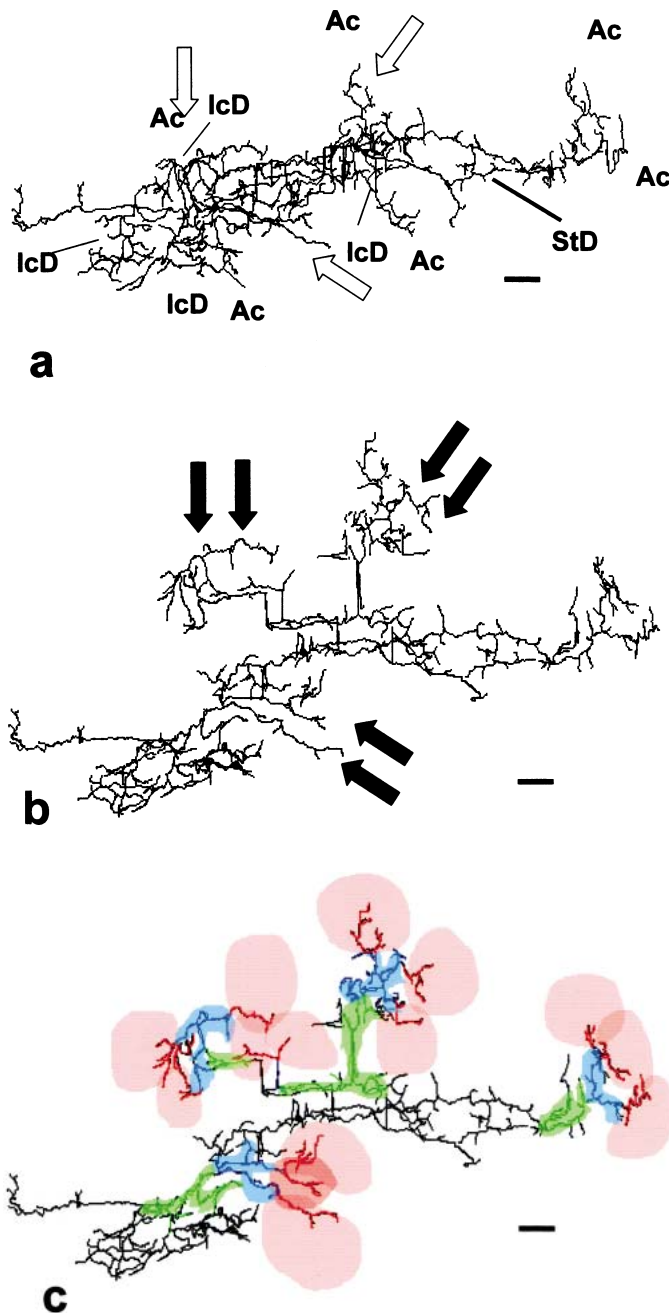


Fig. 4 Three-dimensional view of PGP 9.5 immunoreactive fibers around the terminal secretory portion of the submandibular gland. (StD: striated duct; IcD: intercalated duct; Ac: acinus). Overall features of fiber networks around a terminal secretory portion viewed from different angles (b: 90° rotation from a). Double black arrows indicate single fibers on each terminal acinus after rotation. A single white arrow indicates the complicated fiber appearance before rotation. c. Under the microscope the acinus, intercalated duct and striated duct are superimposed. Terminal secretory portion area was identified and color coded. Red area denotes acinus parts, the blue area intercalated duct parts (included granulated duct parts), and green area striated duct parts.

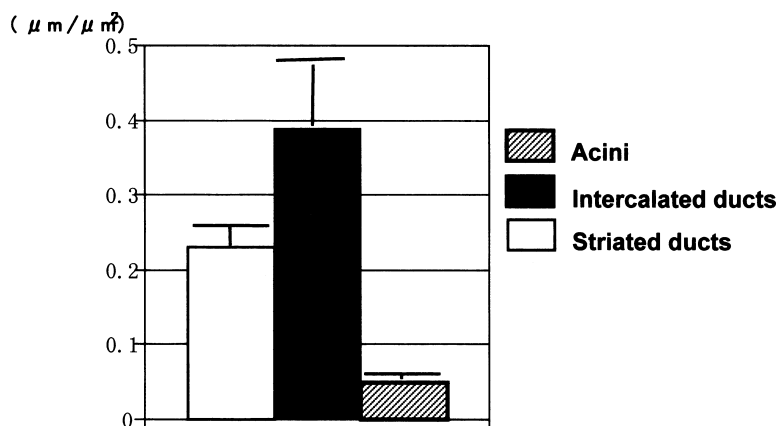


Fig. 5 Comparison of nerve fiber densities among each part of the terminal secretory portion.

In the secretory endopieces, the densities of PGP 9.5 immunoreactive terminals as measured by the computer-aided analysis system were in three areas ; the striated: 0.23 ± 0.021 , the intercalated: 0.39 ± 0.094 , and the acini: $0.05 \pm 0.010 \mu\text{m}/\mu\text{m}^2$. The density of the PGP immunoreactive fibers was most abundant around the intercalated ducts, moderately abundant around the striated ducts, and sparsest around the acinus (Fig. 5).

DISCUSSION

Our present results indicated that many fiber bundles divided from the lingual nerve were distributed over the surface of the submandibular duct, strongly suggesting that almost all such fibers might be supplied from the pathway along the submandibular duct. Some early studies found that many ganglia (periductal ganglia) were observed in the bundles.¹⁾ Our present findings also supported those findings. The presence of periductal ganglia suggested that some populations of fibers in those bundles belonged to an autonomic nervous system. In the hilus of the submandibular gland, the main secretory duct was divided into several interlobular ducts which then entered into the lobules. Fiber bundles were further divided into finer bundles and enter into the lobules together with the interlobular ducts.

Our present three-dimensional results clarified the differences in fiber density among each area of the secretory terminal portion, and indicated that the intralobular duct system together with the striated and intercalated ducts had many more fibers than the acinus. Early studies suggested that the acinus, a functional unit in the production of saliva, was subject to major innervation¹⁶⁻¹⁸⁾. An early ultrastructural study also demonstrated that no nerve terminals were found in regions near the striated duct, but could be observed near the acinus and the intercalated duct¹⁹⁾. Our findings, however, were contrary to these early results. This discrepancy may be caused by technical differences in visualizing the nerve terminals in the thick section.^{20,21)}

Unfortunately, there is no information yet about the reason for dense innervation to the duct system in terms of its function. Recently, however, the existence of granulated convoluted tubules (GCT) has been suggested. These ductal segments are specific submandibular structures connecting the intercalated to the striated ducts.²²⁾ GCT produce and release various peptides such as epidermal growth factor and NGF.^{23,24)} From this point of view, the dense innervation

could effect control of the release of some neuronal peptides release in salivation.

The other important role of nerve fibers in the salivary gland is to maintain the structure and function of the terminal secretory area. Snell *et al.*²⁵⁾ have reported that surgical denervation of the parasympathetic nerves induced marked atrophy in the submandibular gland, suggesting that these nerves contributed to acinar cell survival in the submandibular gland. It has been suggested that the intercalated duct supplies also acinar cell progenitors that are displaced into the acinus.²⁶⁾ In light of this important role, it might be expected that the dense innervation would appear around the ductal structure.

The present study indicated that innervation in the acinus was much less apparent than that in other parts of the terminal secretory portion. It could be considered that areas of less innervation might have suffered more severe damage from denervation.

On the other hand, early morphological studies provided evidence that salivary gland epithelium produces nerve growth factor (NGF).^{27,28)} Our result showing that NGF positive cells were found in the epithelial cells of the duct system, i.e., that epithelial cells accompanied by dense innervation were NGF-immunopositive in character, suggested that epithelial NGF might play a role in the induction of innervation.

The relatively high density of nerve terminals in the intercalated duct suggests that the duct systems probably play important functional and morphological roles.

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