Vagal afferent projections from the pharyngeal jaw of the cichlid Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

We identified vagal innervation in the pharyngeal tooth and jawbones of Nile tilapia through macroscopic observations and immunohistochemistry. We also revealed the apposition of the nerve and osteoclasts in the pharyngeal jaw, suggesting the possibility of neuronal regulation for bone remodeling. However, the central projection from the vagal nerve, which innervates the pharyngeal jaws, remains unknown. To determine the projection of the vagus nerve in the brain, we applied carbocyanine dye (DiI) into the vagus nerve, revealing DiI-labeled neurons in the caudal vagal ganglion. The labeled fibers of the neurons were then traced to the vagal lobe, revealing that they branched and ran dorsally before terminating in a band-like pattern. Meanwhile, the labeled fibers running ventral to the vagal lobe were directed toward the dorsal motor nucleus of the vagus and did not have a definite terminal structure. The vagus nerve innervates the pharyngeal jaw, mainly projects to the vagal lobe, where it receives gustatory information. Pharyngeal tooth-derived sensory information might occur during occlusion and be processed precisely for determining the regurgitation and swallowing of prey.

Key words: tilapia; pharyngeal jaw; vagus nerve; brain; vagal lobe.

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Ethical approval: the study was conducted under the guidelines of the official Japanese regulations for research on animals and of the Yokohama City University (F-A-22-002).

Availability of data and materials: the datasets used and/or analyzed during the current study are available upon reasonable request from the corresponding author.

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Introduction

Cichlids have a pharyngeal jaw in addition to the oral jaw. Similar to the oral jaws, the pharyngeal jaws consist of superior (sPJ) and inferior (iPJ) portions that work together to chew the prey captured by the oral jaws. During this time, the pharyngeal teeth in the sPJ and iPJ are controlled by the masticatory muscles to chew and break the prey into small pieces for swallowing.[1] Meanwhile, although cyprinids also have pharyngeal jaws, their configuration differs from that of cichlids. The upper wall of the pharynx has a masticatory plate that contains a rich distribution of taste buds, and the pharyngeal jaw is located below it.[2] Thus, in cyprinids, the masticatory plates replace the sPJ in cichlids. These anatomical features are suitable for foraging as it allows cyprinids to suck mud and stones and sort food in the pharynx.[3]

We recently identified the nerve entering the bony foramen of the pharyngeal jaw in the Nile tilapia as the vagus nerve and morphologically demonstrated the apposition of its nerve fibers and endings to osteoclasts within the iPJ.[4] In zebrafish and Nile tilapia, the vagus nerve divides into several branches, enters the pharyngeal jawbone, and terminates within the pulp of the pharyngeal teeth.[4,5] Although the function of the peripheral nerves within the pharyngeal tooth pulp in fish remains unknown, it is presumed that sensory information received by the nerves within the pharyngeal tooth pulp during occlusion is transmitted to the central nervous system and used for mastication behavior.

In mammals, the peripheral nerves that receive sensation during occlusion are distributed in the periodontal ligament.[6] Additionally, the pulpal nerve contains the autonomic innervation of blood vessels and sensory nerves that receive sensation during occlusion.[7] In contrast, the periodontal ligament has no counterpart in fish. Therefore, it is possible that sensory information derived from the pharyngeal teeth is transmitted to the central nervous system by the peripheral nerves distributed in the dental pulp.[4,5]

It remains unknown which regions of the brain receive information from the peripheral nerves innervating the pharyngeal jaw. Thus, this study evaluated the regions of the brain that receive information from the vagus nerve by administering a fluorescent carbocyanine dye (DiI) into a branch of the nerve. Surgical tracer injections *in vivo* are considered highly invasive procedures in experimental animals, and approaching the pharyngeal jaw is difficult. This results in a substantial number of sacrificed experimental animals. Alternatively, non-surgical experiments using fixed specimens were planned. Labeling experiments with DiI, which can identify nerve tissue after fixation, were first performed as a pilot study.

Materials and Methods

Animals

Male and female Nile tilapia (*Oreochromis niloticus,* Cichlidae, Teleostei) (n=10, 7.5-10.5 cm in standard length) were obtained from the Tokyo University of Marine Science and Technology. The original study was performed under the guidelines of the official Japanese regulations for research on animals and those of our institution (Yokohama City University, F-A-22-002). The fish were anesthetized by immersing in fresh water containing 150 mg/L of 3-aminobenzoic acid ethyl ester (MS222; Sigma-Aldrich, St. Louis, MI, USA). They were then transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Immunohistochemistry for acetylated tubulin

Two animals were decalcified using the Marse method solution (135-17071, FUJIFILM Wako, Osaka, Japan) for 2-3 weeks. They were then cut in 40 µm frontal cryosections and used for single acetylated tubulin (mouse monoclonal antibody, T6793; Sigma-Aldrich) immunohistochemistry as previously reported.[4] To visualize acetylated tubulin immunoreactivity, the sections were immunoblocked in blocking solution (0.1 M phosphate buffered saline: PBS containing 0.3% Triton-X-100 and 5% normal goat serum) for 1 h, then incubated in the primary mouse antibody for acetylated tubulin (1:400) at 4°C for 2-3 overnight. After washing with 0.1 M PBS, sections were incubated in the secondary biotinylated anti-mouse goat antibody (1:200; Vector Laboratories, Burlingame, CA, USA) for 1.5 h at room temperature. The sections were then washed with 0.1 M PBS and reacted with a solution of the avidin-biotin complex labeled with horseradish peroxidase (Elite ABC kit; Vector Laboratories) for 1.5 h at room temperature. After three 10 min washes in 0.1 M PBS, the sections were reacted with 0.1% diaminobenzidine containing 0.04% nickel ammonium sulphate.

Confirmation of specificity for immunoreactivity

The secondary antibody controls for the immunostaining experiments were performed by omitting the primary antibody. Although some specimens revealed a slightly high background staining image in hematopoietic cells and connective tissues, the antibody used in this study reacted specifically with neural tissue.[8] Acetylated tubulin immunostaining confirmed a positive image in some cells of the tooth germ.[9]

DiI tract tracing experiments

After perfusion, the heads were dissected, and using a stereo microscope (Stemi DV4 Spot; Carl Zeiss Microimaging GmbH, Göttingen, Germany) confirmed that the vagus nerve roots innervated the pharyngeal jaw. We then traced the pharyngeal rami of the vagus nerve running into the foramina of the pharyngeal jawbones.[4]

In the eight fixed head block, DiI (Molecular Probes, Eugene, OR, USA) was administered into the vagus nerve using a fine insect pin under a stereomicroscope. The head block was sealed with 5% gelatin and incubated in 4% PFA at 37°C for 8 months or 1 year. During incubation, DiI diffusion in the vagal ganglion was confirmed in one case, and an image was obtained under fluorescence stereomicroscope that was equipped with an appropriate filter (AXIO Zoom V16; Carl Zeiss Microimaging GmbH). The eight brains and one ganglion were then immersed overnight in 0.1 M phosphate buffer (PB, pH 7.4) containing 20% sucrose followed by embedding of OCT compound (Sakura Finetechnical Co., Tokyo, Japan) and flash freezing by immersion in n-hexane (-60°– -50°C). The blocks were then transversely or sagittally cut into 40 µm thick cryosections[10] and observed under a fluorescence microscope equipped with a Texas red filter (DM-RE; Leica Microsystems, Wetzlar, Germany). DiI-labeled sections were photographed under a fluorescence microscope followed by Nissl staining.

Photographs

Macroscopic photographs were obtained by a digital camera (Nikon D750, Medical Nikkor 120 mm). All serial sections were observed under a light microscope (DM-RE, Leica Microsystems, Germany). Immunohistochemical micrographs were also obtained using digital cameras (DFC290 and DMLD; Leica Microsystems) mounted on a light microscope. The images were adjusted for contrast and brightness to match the real images using an image software (Adobe Photoshop 5.5, San Jose, CA, USA).

Results

Nerve distribution in the pharyngeal jaw: immunostaining with acetylated tubulin antibody

We previously reported the distribution of nerves within the iPJ of tilapia.[4] The gross anatomy shows the nerve bundles innervating the sPJ[4] (Figure 1A). However, the distribution of nerves within the sPJ remains unknown. Acetylated tubulin antibody was used to observe the distribution of peripheral nerves within the sPJ (Figure 1 B,D). Acetylated tubulin-positive nerve bundles entered the bone *via* the bony foramen from the dorsal direction, branched off, and coursed ventrally toward the pharyngeal tooth (Figure 1 A,B). Additionally, nerve bundles traveled within the pulp of the pharyngeal tooth and terminated there (Figure 1D). Other areas that seemed to correspond to the vascular wall as well as some nerve endings terminating in the medullary cavity were noted as previously reported[4] (*data not shown*). Except for the location and number of bony foramina into which the nerve bundles entered, the morphological characteristics of the nerve distribution within the sPJ were similar to those in the iPJ[4] (Figure 1 A,C,E). These findings suggest that the nerve endings within the pulp of the pharyngeal teeth primarily communicate with nerve bundles entering the pharyngeal jawbone (Figure 1 B-E).

Central projection after DiI labeling with the vagus nerve innervating the pharyngeal jaw

DiI was injected into vagus nerve bundles distributed in the pharyngeal jaw (Figure 1 F-I). The labeled nerve bundles in the sPJ and iPJ formed a common trunk proximal to the ganglion (Figure

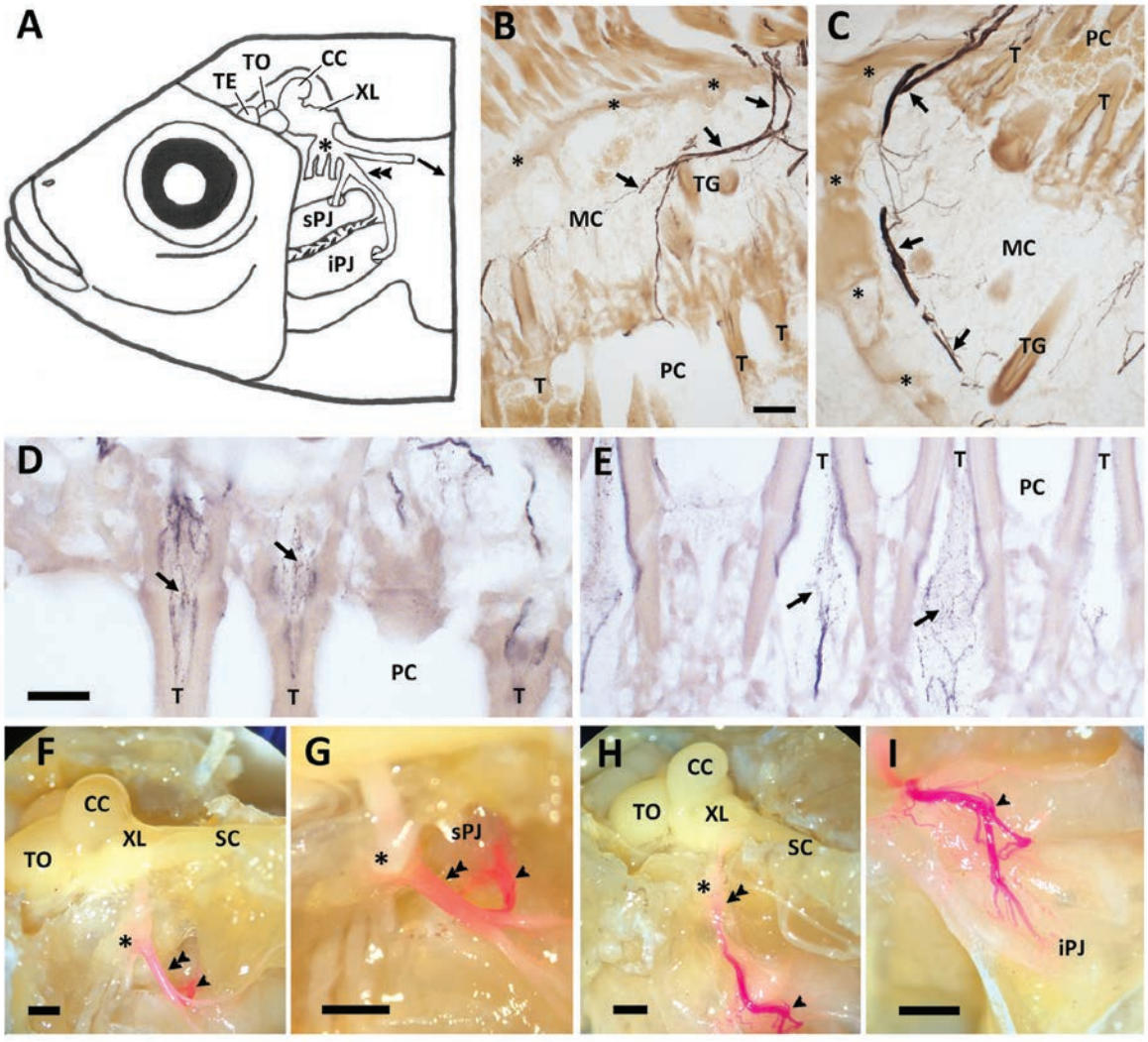

Figure 1. Vagal innervation in the pharyngeal jaws of Nile tilapia and DiI injection sites. **A**) Schematic drawing of the innervation in the pharyngeal jaws; double arrowheads showing the bifurcation of nerves innervating the superior and inferior pharyngeal jaws; asterisk indicates ganglion; arrow showing the running direction of the visceral branch of the vagus nerve. **B**,**C**) Acetylated tubulin-positive nerve fibers entering the superior (**B**) and inferior (**C**) pharyngeal jaw (arrows); asterisks put on base of the pharyngeal jawbones. **D**,**E**) Acetylated tubulin-positive fibers terminating in the superior (**D**) and inferior (**E**) pharyngeal tooth pulp (arrows). **F**,**G**,**H**,**I**) DiI injection sites of the nerve innervating the superior (**F**,**G**) and inferior (**H**,**I**) pharyngeal jaws (arrowhead); higher magnification images of the injection sites (**G**,**I**); double arrowheads showing the bifurcation of nerves innervating the pharyngeal jaws; asterisk indicates ganglion. CC, corpus cerebelli; iPJ, inferior pharyngeal jaw; MC, medullary cavity; PC, pharyngeal cavity; SC, spinal cord; sPJ, superior pharyngeal jaw; T, tooth; TE, telencephalon; TG, tooth germ; TO, tectum opticum; XL, vagal lobe. Scale bars: B,C) 100 μm; D,E) 50 μm; F-I) 1 mm.

1 A,F,G,H,I). Using a fluorescence stereomicroscope, a nerve bundle was labeled centrally from the injection site (Figure 2 A,B), and DiI positivity was also noted in the proximal nerve bundle that connected the ganglion to the vagal lobe (Figure 2 A,B).

Three distinct spindle-shaped or round structures were observed in the Nissl staining of the ganglion (Figure 2C). And the spindle-shaped or round structures contained clusters of neurons surrounded by neuropiles. After confirming DiI labeling, serial sections showed that labeled cells were distributed in the caudal ganglion region (Figure 2 C-F). As DiI labeling was performed in the caudal nerve root, the labeled cells were confined caudally, reflecting a rostro-caudal topographical pattern (Figure 2 C-F). Labeled central projection fibers of labeled ganglion cells entered the vagal lobe from the lateral side (Figure 3 A,B). The labeled fibers further branched and coursed dorsally before terminating as a band-like area within the vagal lobe (Figure 3 A-D). In Nissl staining, the vagal lobe exhibited a distinct layered structure defined by the darkly stained cell bodies of neurons (Figure 3 A,C). This feature was particularly well observed in the superficial layers (Figure 3C). Comparison with Nissl-stained samples confirmed that the striped projections across the gap within the vagal lobe were DiI-labeled nerve endings (Figure 3 C,D). The distinct layered projection pattern to the vagal lobe was observed extending from the caudal region to approximately the anterior two-thirds. In this way, rostro-caudal topographical projection was also observed in the vagal lobe.

Labeled fibers running ventral to the vagal lobe were also noted (Figure 3 A,B). Although the definite terminal structure was unclear, they were directed toward the dorsal motor nucleus of the vagus, which innervates the pharyngeal muscles (Figure 3 A,B). Except for cells labeled within the ganglia, no observations were made for labeled cells in the brain. DiI labeling revealed that nerve bundles distributed in the pharyngeal jaw transmitted information *via* ganglion cells to the vagal lobe in the medulla oblongata (Figure 3 E). In these experiments, no major differences in the projection patterns in the vagal lobes were observed in the labeled cases.

Discussion

This study revealed that the vagus nerve, which innervates the pharyngeal jaw, projected mainly to the vagal lobe. Gustatory and general visceral information is sent to the vagal lobe.[11,12] The nerve bundle entering the bony foramen of the pharyngeal jaw contains the intrapulpal nerves of the pharyngeal teeth, which also ascend to the vagal lobe.[4] The information seems to originate from pharyngeal tooth-derived sensory information, which is captured during pharyngeal jaw occlusion. In the epidermis of the cave-dwelling loricariid catfish *Astroblepus pholeter*, denticles (which are homologous to teeth) are innervated by peripheral nerves and receptive to mechanical stimuli.[13] This indicates that nerves within the pulp of the pharyngeal teeth may be responsible for the transmission of mechanosensory information.

Taste buds are distributed in the epithelium around the pharyngeal tooth in Nile tilapia.[14] Therefore, in addition to sensory information from the dental pulp, it is possible that the labeled nerve bundles in this study might contain fibers that transmit gustatory information. Additionally, as the entire nerve bundle was labeled with DiI, nerve fibers that transmit gustatory sensation and those that innervate the dental pulp may not have been labeled separately. Therefore, it was not possible to precisely determine the origin of the labeled endings that project to the vagal lobe.

In Nile tilapia, although the central neural circuits responsible for gustatory and visceral sensation have been reported,[11,12] there

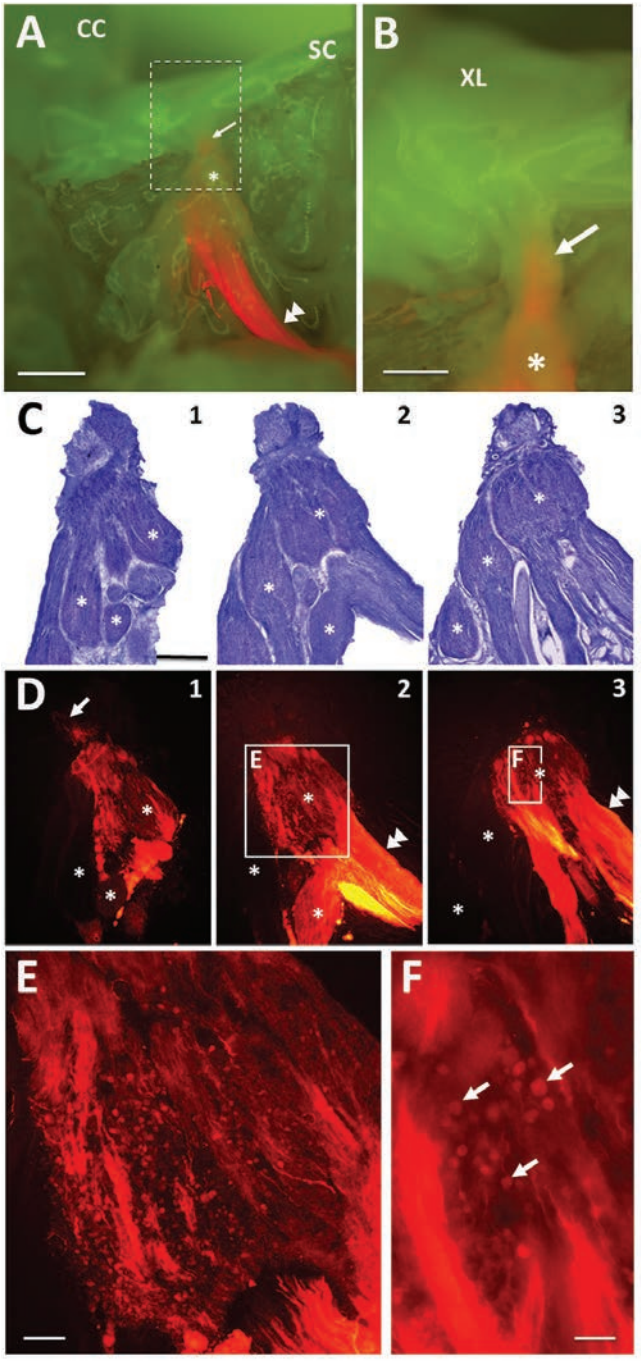

Figure 2. DiI-labeled ganglion after DiI injection into the vagus nerve innervating to sPJ. **A,B**) Lateral view of the fluorescent stereomicroscopic images of the DiI-labeled vagal ganglion; left is the rostral direction; asterisk and double arrowhead indicate the DiI-labeled ganglion and distal nerve root, respectively. **B**) Higher magnification of the dashed lined box in (**A**); arrow shows the labeled proximal vagus nerve root. **C,D**) Sagittal sections of the vagus ganglion (asterisks) with Nissl-stained (**C**) and DiI-labeled (**D**) sections; numbered panels correspond to the same samples with different visualizations, Nissl-stained samples, and DiI-labeled samples (**C,D1-3**); panels are presented mediolaterally from no. 1 to no. 3; note that most posterior ganglion was clearly labeled. **E,F**) Higher magnification images showing DiI-labeled neurons. **E**) Higher magnification of the white-lined box in (**D2**). **F**) Higher magnification of the white-lined box in (**D3**); several labeled neurons were noted in **F** (arrows). CC, corpus cerebelli; SC, spinal cord; XL, vagal lobe. Scale bars: A) 1 mm; B-D) 0.5 mm; E) 100 μm; F) 50 μm.

are no reports on the central neural circuits that transmit sensory information from the pharyngeal teeth. In zebrafish pharyngeal jaws, nerve fibers from the vagus nerve are present in the pulp of the pharyngeal tooth, but their central projections are unknown.[5] Gustatory information is transmitted to the vagal lobe and ascends to the telencephalon *via* the relay nuclei,[11] while visceral sensations enter the commissural nucleus of Cajal and are transmitted to the vagal lobe and telencephalon also *via* the relay nuclei.[12] Thus, sensory information originating from the pharyngeal tooth may also enter the vagal lobe as well as the gustatory information. The vagal lobe, wherein peripheral nerves that may contain pulp-, taste bud-, and viscera-derived information project to, precisely controls the regurgitation and swallowing of food based on gustatory information, as in the case of goldfish.[3] Thus, it is possible that a similar functionally effective neural circuit is also present in Nile tilapia. Furthermore, projections from the vagus nerve, thought to originate in the palate, to the vagal lobe have been reported in other cichlids, and these projections exhibit a layered structure.[15] The projection pattern was similar to our results, and it may be a common feature among the cichlid species. However, although we were able to observe a band-like, or layered, projection pattern to the vagal lobe in this study, the cellular architecture and corresponding neural circuitry in Nile tilapia based on a layered structure similar to that of the cyprinid vagal lobe remains unknown.[16]

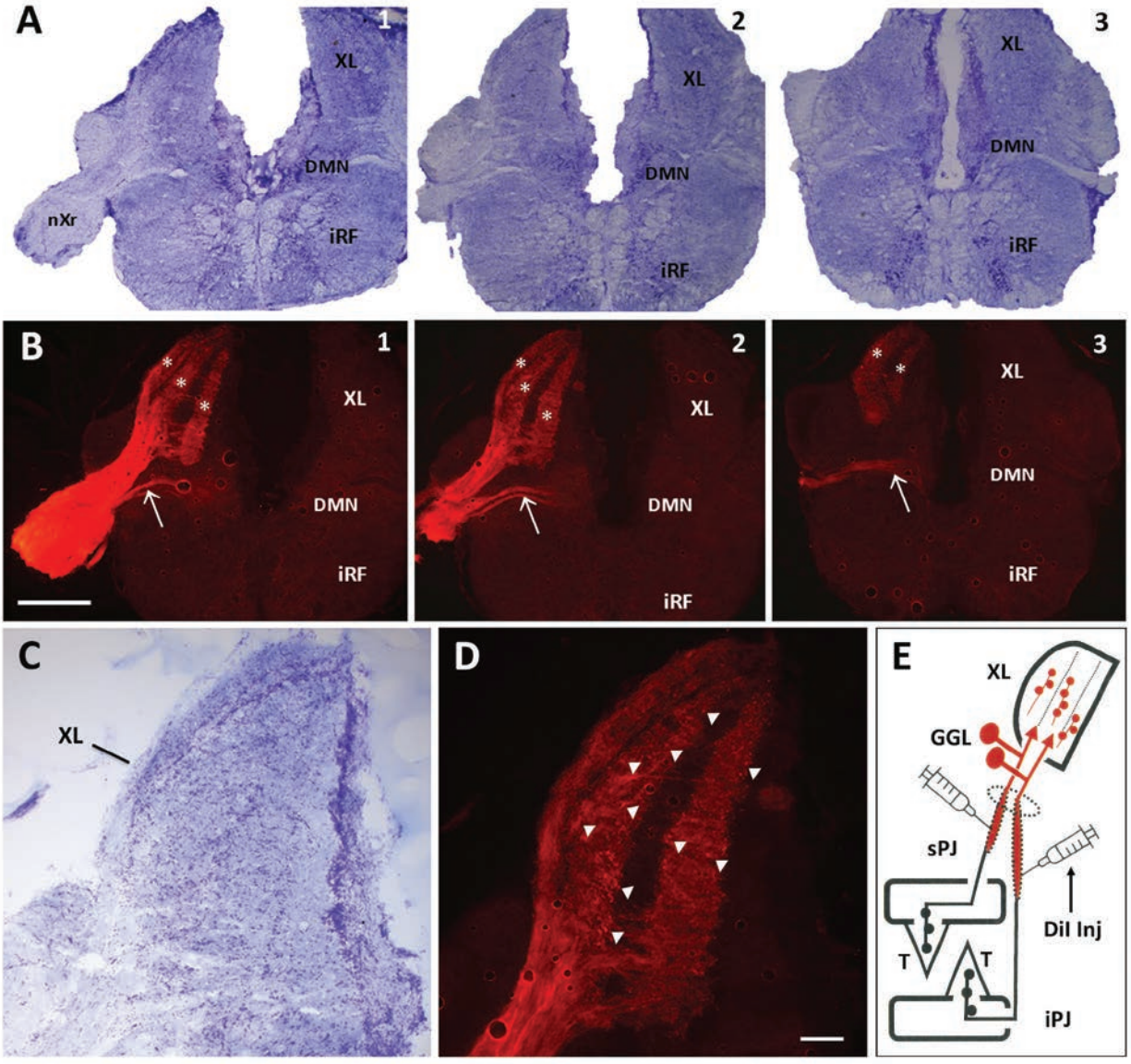

Figure 3. Projections to the vagal lobe from the nerve fibers innervating the sPJ. **A,B**) Frontal sections of the brain at the level of the vagal lobe of Nissl-stained (**A**) and DiI-labeled (**B**) samples; the numbered sections correspond to the same samples with different visualization (**A,B 1-3**), 1-3 organizing in a rostrocaudal direction; asterisks indicate the DiI-labeled fibers projecting to the vagal lobe with a band-like fashoin after DiI injection; arrow showing labeled fibers heading to the dorsal motor nucleus of the vagus. **C,D**) Higher magnification of the vagal lobe; **C**) Nissl stained; **D**) shows the DiI-labeled fibers and terminals; note the fine beaded terminals in the vagal lobe (arrowheads). **E**) Schematic summary of this research; DiI was injected into the nerve fibers innervating the superior and inferior pharyngeal jaws; both DiI-labeled fibers projected to the vagal lobe in a band-like fashion *via* the labeled vagal ganglion (red color); dashed oval indicating the bifurcation of the two nerve fibers. DMN, dorsal motor nucleus of the vagus; GGL, ganglion; Inj, injection; iPJ, inferior pharyngeal jaw; iRF, inferior reticular formation; nXr, vagal nerve root; sPJ, superior pharyngeal jaw; T, tooth; XL, vagal lobe. Scale bars: A,B) 0.5 μm; C,D) 100 μm.

Future clarification of the cellular architecture in response to sensory input to the vagal lobe is warranted to determine the function of the neural circuitry of the vagal lobe in Nile tilapia.

We also considered the possibility of peripheral neuronal control of bone metabolism by showing that nerve fiber bundles and terminals distributed within the pharyngeal jaw of Nile tilapia are in apposition to osteoclasts.[4] Furthermore, parathyroid hormone 4 (PTh4)-expressing neurons in the hypothalamus in zebrafish are involved in the osteogenesis and controlled by the central nervous system.[17] Thus, disruption of these neurons during development inhibits the formation of bone regions, including the pharyngeal jaw.[17] Although the brain is responsible for bone metabolism, it remains unclear whether peripheral nerves interact with osteoclasts within the pharyngeal jaw of the zebrafish. Additionally, it remains unknown whether hypothalamic neurons expressing PTh4 influence pharyngeal jaw osteogenesis and *via* which neural circuits within the brain.[18] Future research should investigate whether PTh4-expressing neurons exist in the hypothalamus in Nile tilapia and whether information from the vagus nerve innervating the pharyngeal jaw communicates with such neurons to gain a better understanding of the neuronal bone metabolism mechanism. Finally, it will be interesting to see how the vagus nerve, which is in apposition to osteoclasts within the pharyngeal jaw, relates to the hypothalamic region as described above.

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