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Research article

Distribution, nature, and origin of CXCL14-immunoreactive fibers in rat parotid gland

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ABSTRACT

The distribution and nature of CXCL14-immunoreactive nerve fibers in salivary glands, especially the parotid gland was immunohistochemically investigated. Furthermore, the origin of parotid CXCL14-immunoreactive nerve fibers was determined by retrograde tracing experiments. CXCL14-immunoreactive nerve fibers were localized in the parotid, submandibular, and sublingual glands, particularly in the parotid gland. Double staining using identical sections revealed that a subpopulation of cells neuropeptide Y (NPY)-containing fibers was immunopositive for CXCL14 in the parotid gland. In the peripheral regions of acinar cells, CXCL14-immunoreactive fibers tended to coexist with NPY; however, perivascular NPY-immunoreactive fibers tended to be immunonegative for CXCL14. Parotid CXCL14-immunoreactive fibers were immunopositive for tyrosine hydroxylase (TH) but immunonegative for choline acetyltransferase and vasoactive intestinal peptide (VIP). After injection of horseradish peroxidase-labeled wheat germ agglutinin (WGA-HRP) in the parotid gland, retrogradely labeled neurons were seen in the superior cervical ganglion (SCG) and otic ganglion. Some of the WGA-immunoreactive somata in the SCG were immunopositive for CXCL14; however, no doubly-labeled somata were noted in the otic ganglion. These results indicate that CXCL14-immunoreactive nerve fibers originate in the SCG, and are sympathetic in nature. The coexistence of CXCL14 with NPY/TH suggests that CXCL14 may be associated with NPY/TH functions as a neuromodulatory chemokine in the parotid gland. The localization of CXCL14 nerve fibers around the acinar cells of the parotid gland indicates its involvement in acinar cell function, but not vasoconstriction.

1. Introduction

Major salivary glands are composed of the parotid, submandibular, and sublingual glands. Among these, the parotid gland is serous in nature, whereas the submandibular and sublingual glands are mixed glands consisting of both serous and mucous cells. The parotid gland is recognized as an important secretory and digestive organ owing to the presence of amylase, and is mainly controlled by the autonomic nervous system [7,9]. The contents and composition of saliva are controlled by sympathetic nerves, while the parasympathetic nerves influence the volume of saliva [4]. Salivation occurs in two stages; one is by the elaboration of isotonic primary saliva by acinar cells, and the second is via the fluxing of electrolytes in the duct system to yield a final hypotonic saliva. The major source of parotid sympathetic innervation originates in the superior cervical ganglion (SCG) [13,14,21]. The neurons in the SCG are immunopositive for tyrosine hydroxylase (TH), a marker of catecholaminergic nerves, and for a number of neuropeptides, such as neuropeptide Y (NPY) and galanin [11,41]. In contrast, the major origin of parotid parasympathetic neurons is the otic ganglion, which contains cholinergic neurons and various kinds of neuropeptides, such as vasoactive intestinal peptide (VIP) and substance P [20,33].

Chemokines fundamentally possess chemotactic activity for leucocytes and lymphocytes. The CXC-type chemokines have one amino acid between two cysteines and are further divided into ELR^+ and ELR^- subtypes depending on the presence of “Glu-Leu-Arg” residues at the N-terminal [23]. CXCL14 is a member of the CXC subfamily and belongs to the ELR^- subtype. CXCL14 was first isolated from human breast and kidney cells, and originally termed BRAK [19]. It is expressed in epithelial tissues and mesenchyme-derived cells in the body [26]. However, it is not expressed in the majority of the head and neck squamous

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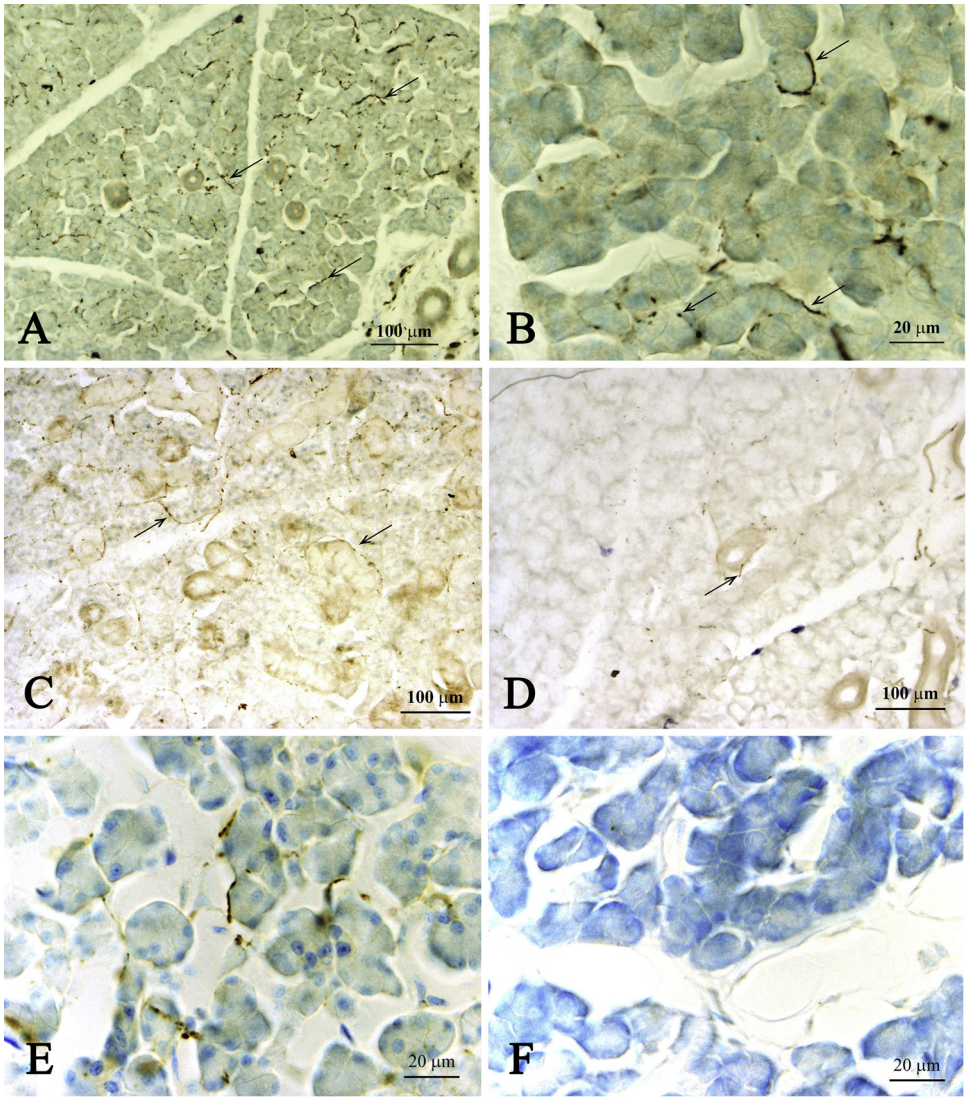

Fig. 1. CXCL14-imunoreactive profiles in the parotid (A, B), submandibular (C), and sublingual (D) salivary glands, and results of pre-absorption tests in the acinar cell region of the parotid gland (E, F). Arrows in A-D indicate CXCL14-immunoreactive nerve fibers. Pre-absorption of the antibody with CXCL14 resulted in disappearance of staining profiles (F). Sections were counterstained with thionin. Bars in A, C, D = 100 μm, and those in B, E, F = 20 μm.

cell carcinomas, and other established human cancer cell lines [12,19]. Several physiological functions of this chemokine have been reported, such as recruitment and maturation of monocyte-derived macrophages, renewal of Langerhans cells in the skin, stimulation of trafficking of activated natural killer cells to the sites of inflammation or malignancy, infiltration of macrophages, and inhibition of angiogenesis [22,27,31,35,36]. Suppression of tumor formation by this chemokine has also been reported [28,39]. Interestingly, CXCL14 is expressed in neuronal and/or glial cells in the central and peripheral nervous systems [3,16,29,32,37,42]. This implies that CXCL14 possesses neuron-associated functions such as a neuromodulator and a regulator of neuronal migration in addition to chemotactic functions for immune cells.

In the present study, we demonstrated the presence of CXCL14-immunoreactive fibers in salivary glands, and the existence of a subpopulation of NPY-immunoreactive fibers in the parotid gland. We further clarified that CXCL14-immunoreactive fibers in the parotid gland originated from the sympathetic SCG.

2. Materials and methods

All animal procedures were carried out under the authority of the Institutional Animal Care and Use Committee of Kanagawa Dental University, employing the guidelines established by the committee (permitted no. 17-015 and 18-036). A total of 14 animals were sacrificed. Seven male Sprague-Dawley rats were deeply anesthetized with sodium pentobarbital (Kyouritsu Seiyaku Corporation, Tokyo, Japan). The animals were then perfused with 0.85% NaCl, and subsequently, with 4% formaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer (PB; pH 6.9). The parotid gland was rapidly dissected; the submandibulars and sublingual glands were also dissected for Supplementary data. The dissected glands were fixed in the same fixative for one or two days at 4 °C. Subsequently, the samples were embedded in 10% gelatin, blocked, fixed for 1 day at 4 °C, and stored in PB. Gelatin-embedded salivary glands were immersed in 20% sucrose and cut into 20 μm-thick sections using a sliding microtome equipped with a frozen stage. The sections ware stocked in 0.1 M PB (pH 7.4) containing 0.9% saline (PBS). Immunostaining was performed according to the methods described previously [42]. Briefly, the sections were washed in PBS overnight, and incubated with rabbit anti-human CXCL14 antibody (500-P237; PeproTech Inc., Rocky Hill, NJ, USA) diluted to 0.5 μg/ml in PBS containing 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (PBS-BSAT) for 24 h at 4 °C. The antibody was purified by affinity chromatography employing an immobilized human

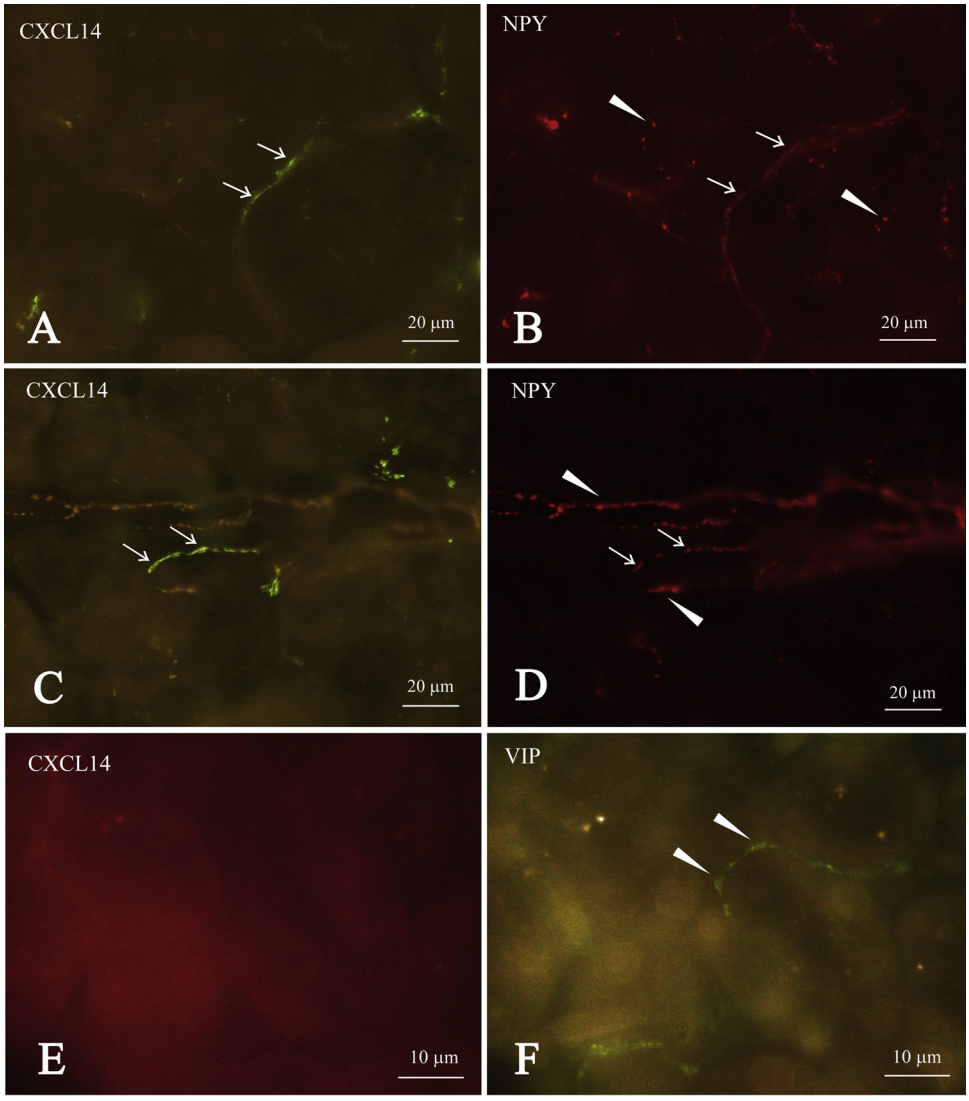

Fig. 2. Double staining of CXCL14 (A, C, E) with NPY in the acinar cell region (B) and perivascular region (D), and with VIP (F) in the parotid gland. Arrows in A-D indicate coexisting nerve fibers. Arrowheads in B, D, and F indicate singly labeled nerve fibers with anti-NPY antibody (B, D) and anti-VIP antibody (F). A and B, C and D, and E and F present identical sections. Bars in A-D = 20 μm, and those in E and F = 10 μm.

CXCL14 matrix according to the manufacturer's instruction. After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (BA-1000; Vector laboratories, Burlingame, CA, USA) diluted to 1:200 in PBS-BSAT for 1 h at room temperature. The sections were then washed again in PBS and incubated with avidin-biotin-horseradish peroxidase (HRP) complex (ABC; PK-6100; Vector Laboratories) diluted to 1:200 in PBS-BSAT for 30 min at room temperature. After a final wash in PBS, the sections were reacted with 0.02% 3, 3'-diaminobenzidine tetrahydrochloride (DAB) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer solution (pH 7.4). Thereafter, the sections were counterstained with thionin and coverslipped using Malinol (Muto Pure Chemicals, Tokyo, Japan). Controls were prepared by omitting the primary antibody during the incubation processes. As an additional control, sections were stained with the antibody pre-absorbed with recombinant human CXCL14 (300-50; PeproTech Inc.; 5 μg/ml). To determine the nature of CXCL14-immunoreactive nerve fibers in the parotid gland, double immunofluorescence staining was performed using sheep anti-NPY serum (AB1583; Chemicon International Inc., Temecula, CA, USA; 1:1,000 dilution in PBS-BSAT), sheep anti-TH serum (AB1542; Chemicon International Inc.; 1:1,000 dilution in PBS-BSAT), goat anti-choline acetyltransferase (CAT) antibody (AB144P; Millipore Corporation, Temecula, CA, USA; 1:100 dilution in PBS-BSAT), and guinea pig anti-VIP antibody (03-16071; American Research Products, Inc., Belmont, CA, USA; 1:50 dilution in PBS-BSAT). Immunoreactivities were visualized by Alexa Fluor 488-labeled donkey anti-rabbit IgG (Abcam, Cambridge, UK) for CXCL14 and Alexa Fluor 555-labeled donkey anti-sheep IgG (Abcam) for NPY and TH. In case of double staining for CXCL14 and CAT, CXCL14 was visualized with Texas red-labeled donkey anti-rabbit IgG (GeneTex Inc., Irvine, CA, USA) and CAT with FITC-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology Inc., Dallas, TX, USA). Doubly stained sections of CXCL14 and VIP were visualized with Texas red-conjugated donkey anti-rabbit IgG (CXCL14; GeneTex Inc.) and FITC-conjugated donkey anti-guinea pig IgG (VIP; Rockland Immunochemicals Inc., Limerick, PA, USA). For triple staining of CXCL14, TH (in this case, we used a mouse monoclonal antibody; 22941; ImmunoStar Inc., Hudson, WI, USA; 1:1,000 dilution in PBS-BSAT) and NPY, CXCL14 was visualized with fluorescein-labeled donkey anti-rabbit IgG (Chemicon International Inc.), TH with rhodamine-labeled donkey anti-mouse IgG (Millipore Corporation), and NPY with biotinylated donkey anti-sheep IgG (Chemicon International Inc.) and aminomethylcoumarin (AMCA) conjugated avidin D (Vector Laboratories).

To determine the origin of CXCL14-immunoreactive fibers in the parotid gland, HRP-labeled wheat germ agglutinin (WGA-HRP) was

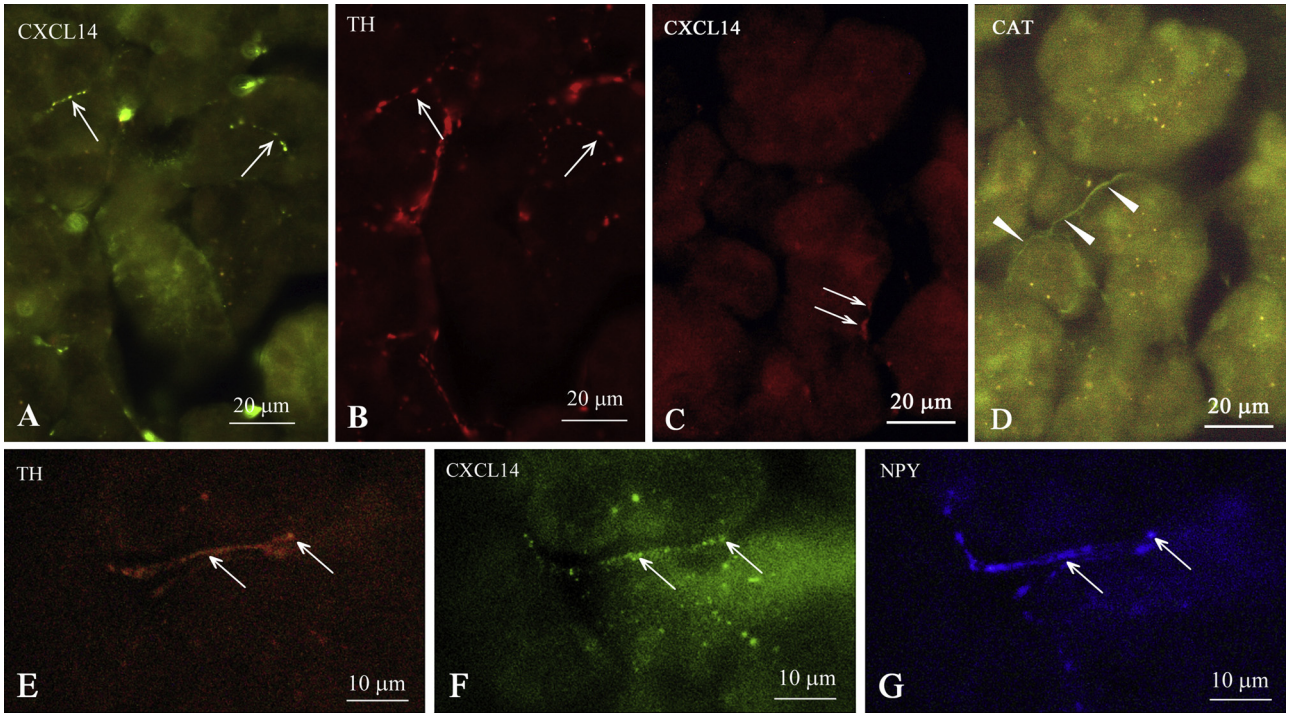

Fig. 3. Double staining of CXCL14 (A, C) with TH (B) and CAT (D) in the parotid gland. Arrows in A and B indicate doubly labeled nerve fibers. Arrows in C indicate CXCL14 immunoreactive fibers and arrowheads in D indicate singly labeled nerve fibers with anti-CAT antibody. Triple staining of CXCL14 (F), TH (E) and NPY (G). Arrows in E, F and G indicate triply labeled fibers. A and B, C and D, and E, F and G present identical sections. Bars in A-D = 20 μm and those in E-G = 10 μm.

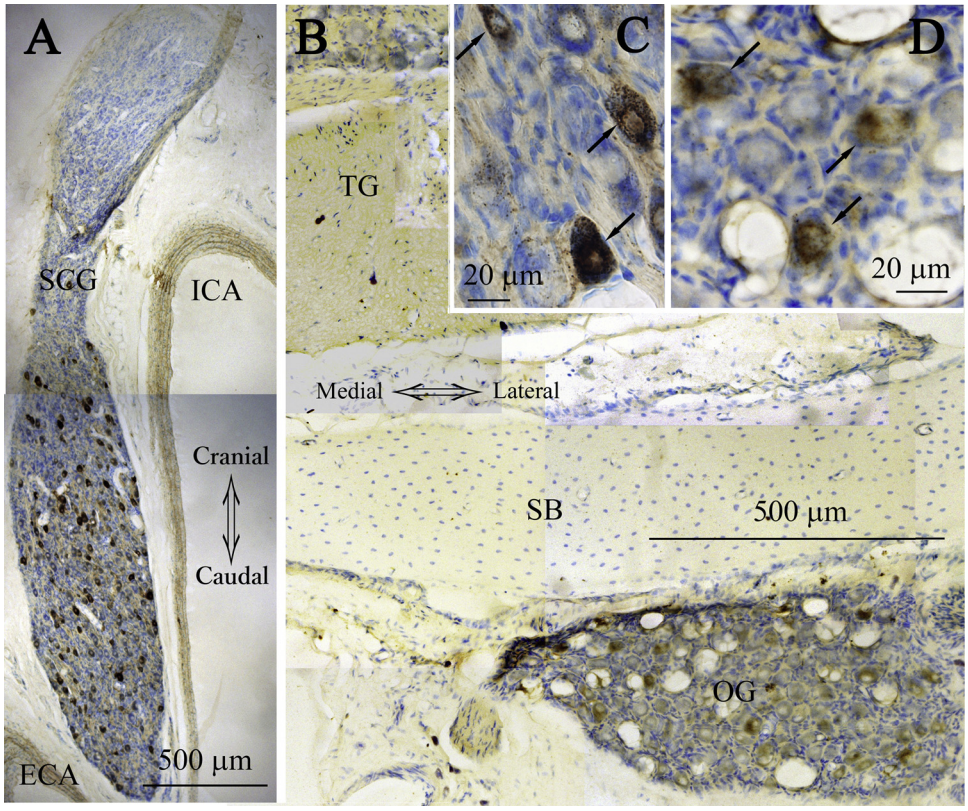

Fig. 4. Retrogradely labeled neurons in the right superior cervical (A, C) and right otic (B, D) ganglia. Arrows in C and D indicate WGA-immunoreactive neurons. Sections were counterstained with thionin. A and B are montages showing the association of the superior cervical ganglion with the internal and external carotid arteries, and the association of the otic ganglion with the trigeminal ganglion and sphenoid bone. Abbreviations: ECA, external carotid artery; ICA, internal carotid artery; OG, otic ganglion; SB, sphenoid bone; SCG, superior cervical ganglion; TG, trigeminal ganglion; Bars in A and B = 500 μm, and those in C and D = 20 μm.

used as a retrograde neuron tracer in seven animals. After deep anesthesia with sodium pentobarbital (Kyouritsu Seiyaku Corporation), the right parotid gland was surgically exposed by a skin incision 1 cm lateral to the midline, and parotid tissue was identified as a yellowish gland under the angle of the mandible. WGA-HRP (10 μl) was injected in four positions in the parotid gland (2.5 μl in each position) using a Hamilton micro-syringe. After the injections, the skin incision was closed with silk sutures (Matsudaika Kogyo Co. Ltd. Tokyo, Japan). One week later, the animals were anesthetized again with sodium pentobarbital (Kyouritsu Seiyaku Corporation) and transcardially perfused

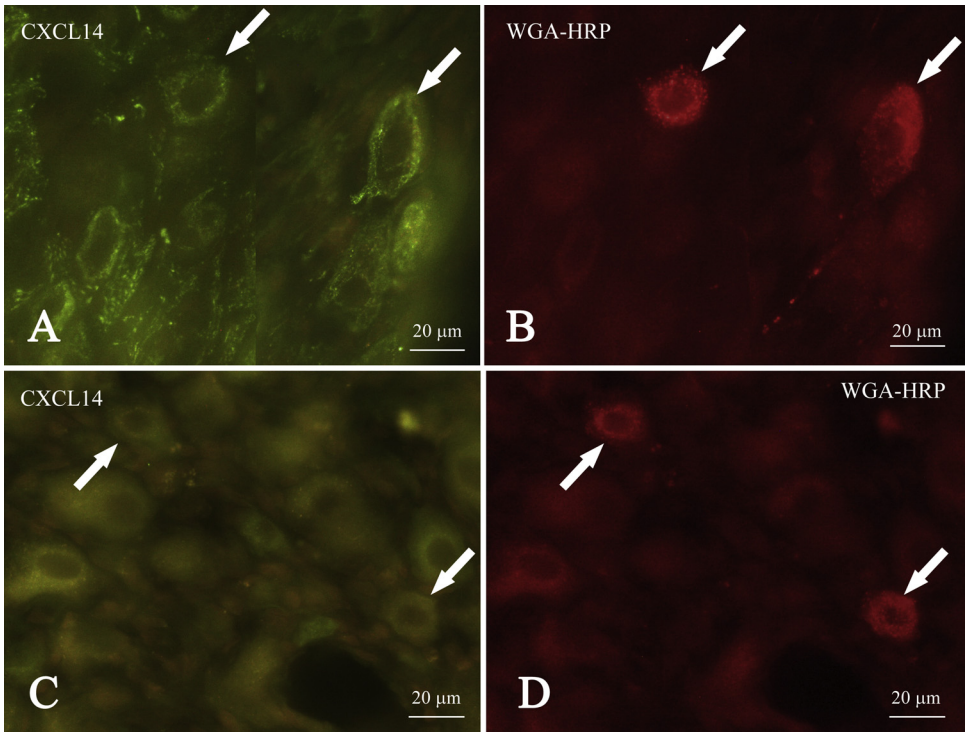

Fig. 5. Double staining of CXCL14 and WGA in the right superior cervical (A, B) and right otic (C, D) ganglia. Arrows in A and B indicate doubly labeled neurons with anti-CXCL14 (A) and anti-WGA (B) antibodies. Arrows in C and D indicate retrogradely labeled neurons (D) but not immunopositive for CXCL14 (C). A and B, and C and D present identical sections. Bars in A-D = 20 μm.

with 0.85% saline and the afore mentioned fixiative. The right parotid gland, right and left SCG with common, internal, and external carotid arteries, and right and left otic ganglia with the trigeminal ganglia and sphenoid bone were dissected. The right and left SCG were separately embedded in gelatin; in addition, a tissue block of the right and left otic ganglia with the trigeminal ganglia and sphenoid bone was also embedded in gelatin after decalcification with EDTA. The SCG was cut into sagittal sections, while the otic ganglia was cut along the coronal plane. The sections were immunohistochemically stained with goat anti-WGA antibody (AS-2024; Vector Laboratories; 1:1,000 dilution in PBS-BSAT) and visualized by biotinylated horse anti-goat IgG (BA-9500; Vector Laboratories), ABC, and DAB reactions. For fluorescence double staining with CXCL14 and WGA, CXCL14 was visualized with Alexa Fluor 488-labeled donkey anti-rabbit IgG (Abcam) and WGA with rhodamine-conjugated donkey anti-goat IgG (Millipore Corporation). The SC and otic ganglion were identified as described previously [1,2].

3. Results

CXCL14-immunoreactive fibers were observed mainly in the peripheral regions of acinar cells and around vascular vessels; however, a few were seen around secretory ducts in the parotid gland (Fig. 1A, B). In the submandibular gland, CXCL14-immunoreactive fibers tended to be associated with granulated ducts (Fig. 1C). On the other hand, only a few CXCL14-immunoreactive fibers were seen in the sublingual gland (Fig. 1D). These immunoreactive profiles were abolished by pre-absorption of the antibody with recombinant CXCL14 (Fig. 1E, F). Sections processed from the secondary antibody showed no staining profiles (not shown).

Double staining with CXCL14 with NPY indicated that most of CXCL14-immunoreactive fibers around the parotid acinar cells were immunopositive for NPY (Fig. 2A, B); nonetheless, NPY-immunopositive and CXCL14-immunonegative fibers were also seen (Fig. 2A, B). Non-coexistence was more prominent around blood vessels, where most of the NPY-immunoreactive fibers were immunonegative for CXCL14 (Fig. 2C, D). Furthermore, all the VIP-immunoreactive fibers were immunonegative for CXCL14 (Fig. 2E, F). Similarly, CXCL14-immunoreactive fibers surrounding acinar cells were immunopositive for TH (Fig. 3A, B); however, the CAT-immunoreactive fibers were immunonegative for CXCL14 (Fig. 3C, D). Triple staining of CXCL14 with NPY and TH indicated that some fibers surrounding acinar cells were immunopositive for CXCL14, NPY and TH (Fig. 3E–G).

Retrograde labeling experiment using WGA-HRP indicated that some ganglion cells in the only ipsilateral SCG and otic ganglia were immunopositive for WGA (Fig. 4). Some of the WGA-immunoreactive cells in the SCG were immunopositive for CXCL14 (Fig. 5A, B), whereas in the otic ganglion, the WGA-immunoreactive cells were immunonegative for CXCL14 (Fig. 5C, D).

4. Discussion

Sympathetic innervation contributes to the production of dense, low volume but enzyme-rich saliva, whereas parasympathetic innervation is involved in the secretion of a large volume of watery serous saliva [4]. The major source of parotid sympathetic innervation originates in the SCG [13,14,21]. NPY has been found in rat [34] and human [17] parotid glands. NPY is thought to play a role in the modulation of glandular secretion [8] and constriction of salivary gland vasculature [24]. The presence of CXCL14-like peptides in NPY fibers around acinar cells in the current study suggests that CXCL14 is involved in the regulation of acinar cell secretion rather than vasculature constriction, in concert with NPY/TH. The subpopulational coexistence of the CXCL14 nervous system has been reported in the somatostatinergic system in mouse alimentary tract [37]. It is worthy to note that CXCL14 coexists with somatostatin, which has secretomotor functions, but not with somatostatinergic inhibitory interneurons [6,37]. Saliva secretion is a complex process orchestrated and/or modulated by a multitude of various neurochemicals present in salivary gland neurons [10,18]. To the best of our knowledge, this is the first study to demonstrate the involvement of CXCL14, in addition to the various neurochemicals reported previously, in the functions of the parotid gland.

CXCL14-immunoreactive fibers in the parotid gland corresponded to a subpopulation of NPY-containing fibers that were immunopositive for TH, suggesting that the CXCL14-immunoreactive fibers were the sympathetic nature. Retrograde tracing experiments confirmed the sympathetic nature of CXCL14-immunoreactive fibers in the parotid

gland. The functional characteristics of CXCL14 as a neuromodulator and/or neurotransmitter remains elusive. Nevertheless, the existence of CXCL14 in neurons has been demonstrated in the hypothalamic [42], hippocampal GABAergic [3], and somatostatinergic neurons [37]. CXCL14 modulates and maintains the expression levels of TH in mesencephalic dopaminergic neurons [40], though the neurons in the SCG are generally noradrenergic; thus, we believe that the peptide might have similar functions in the neurons of the SCG. In GABAergic neurons, CXCL14 inhibits the tonic and phasic effects of synaptically-released GABA [3]. Interestingly, CXCL12, another CXC-type chemokine, has the opposite functions in GABAergic neurons, where it magnifies the effects of the released GABA [3]. In addition to the above mentioned functions, CXCL12 stimulates the release of glutamate from astrocytes and affects the synaptic activity of the neurons [5]. Furthermore, it reduces calcium oscillation in hippocampal neurons [25]. To date, the specific receptors for CXCL14 are not known, and it is proposed that the contradictory effects of CXCL14 and CXCL12 are exerted through the same receptor, CXCR4 [38]. Although CXCR4 expression in the parotid gland is not known, further study about CXCR4 may evaluate parotid CXCL14 functions. Other chemokines are also known to act as chemotactants as well as neuromodulators. CXCL8 reduces calcium currents in cholinergic neurons [30]; it also modulates calcium transients, enhances synaptic activity, and suppresses the induction of long-term depression in cerebellar Purkinje cells [15]. Taking together, these findings indicate that CXCL14 may serve as a neuromodulator and/or a neurotransmitter. However, we could not deny the possibility that CXCL14 released with NPY in the parotid gland contributes to the recruitment of immune cells as demonstrated in the peripheral organs [22,31,35].

The coexistence of CXCL14 with various substances has been reported; CXCL14 coexists with vasopressin/oxytocin in the hypothalamus [42], with GABA in the hippocampus [3], and with somatostatin in the alimentary tracts [37]. The present study indicated the coexistence of CXCL14 with NPY. Although the complete functions of CXCL14 are not known, the diversity of the substances that coexist with this peptide suggests that CXCL14 may be associated with other additional functions, such as the regulation of the physiological states of neurons. The association of CXCL14 with neuron development has been demonstrated previously [16,29]. However, future studies elucidating these functions are warranted.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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