Morphological Interrelationship Between Astrocytes and Nerve Endings in the Rat Spinal Trigeminal Nucleus Caudalis

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Summary: It has been reported that the spinal trigeminal nucleus caudalis (Sp5C), which receives nociceptive information from the oro-facial regions, has four laminae. To clarify the role of glial cells in the transmission of the nociceptive information, the present study was conducted to examine the detailed distribution of astrocytes in each lamina and also to investigate a morphological interrelationship between the astrocytes and nerve endings in the rat Sp5C. After the preparation of the serial cryostat sections, immunohistochemistry for glial fibrillary acidic protein (GFAP) was employed to identify the astrocytes, and immunohistochemistry for substance P (SP), calcitonin gene-related peptide (CGRP), was used for the nerve endings. We also employed double-labeling immunofluorescence and electron microscopic immunohistochemistry for the GFAP/SP or GFAP/CGRP. GFAP-positive reactions were observed in all laminae of the Sp5C, and SP- or CGRP-positive nerve endings were observed in the lamina I and II. Additionally, we clarified the presence of GFAP/SP- or GFAP/CGRP-positive reactions by the double-labeling immunofluorescence and demonstrated the morphological interrelationship between the astrocytes and nerve endings by the double-labeling electron microscopic immunohistochemistry. These findings suggest that astrocytes might play some roles in the transmission of nociceptive information from the oro-facial region.

Spinal trigeminal nucleus caudalis (Sp5C) plays important roles in the transmission of the nociceptive information from the oro-facial regions^{1–4}. The Sp5C is divided into four laminae (I–IV) from the outside layer to the layer on the inside, sequentially⁵. Additionally, lamina I and II of the Sp5C receive the neuronal inputs of the primary afferent nerve endings, which contain neurotransmitters such as calcitonin gene-related peptide (CGRP) and substance P (SP)^{6,7}.

Recently, it has been reported that glial cells may be involved in the transmission of the nociceptive information⁸. In the Sp5C, Hajos *et al.* demonstrated the presence of astrocytes using the immunohistochemistry (IHC) for glial fibrillary acidic protein (GFAP)⁹ that is a specific marker for astrocytes¹⁰. However, the detailed morphological investigations between the astrocytes and the nerve endings have not yet clarified.

In the present study, IHC for GFAP was employed to investigate the detailed distribution of astrocytes in each lamina of the Sp5C. Furthermore, double-labeling immunofluorescence (IF) and electron microscopic immunohistochemistry (EMIHC) for GFAP/SP or GFAP/CGRP was also employed to investigate a morphological interrelationship between the astrocytes and the nerve endings.

Materials and Methods

A total of thirty-two male Wistar rats (8 weeks after birth; purchased from Shimizu laboratory Supplies, Kyoto Japan) were used in the present study. These animals were housed in a temperature-controlled room (23–25°C). They were maintained in individual cages under a light/dark cycle (lights on at 0800, lights off at 2000) and were given free

access to food and water. The present study was conducted according to the Guidelines for Animal Research at Osaka Dental University (ODU) and the National Institute of Health (NIH) Guideline for the Care and Use of Laboratory Animals, and the study was approved by the Animal Research Committee for ODU (approval number; 05-06002).

A. Light Microscopic Investigation

All of the animals were sacrificed by an overdose injection of pentobarbital (50 mg/kg, i.p.; Nembutal®, Dainippon Pharmaceutical Co. Ltd) and immediately were perfused via the left cardiac ventricle with a 0.1 M phosphate-buffered saline (PBS) and a fixative containing 4% paraformaldehyde, which was adjusted to pH 7.4 with 0.1 M phosphate buffer (PB). After the removal of the brain, tissue blocks including the Sp5C were post-fixed in the same fixative for 24 h at 4°C, and then immersed in 20% sucrose in 0.1 M PB for 24 h at 4°C.

Serial, free-floating frontal sections (30 µm in thickness) were prepared with a cryostat (MICROM HM500-OM®, ZEISS) and the following histological procedures were performed.

1. Nissl staining

To investigate the cellular location in the Sp5C, sections obtained from four animals were used for Nissl staining in a 0.1% cresyl violet (Cresyl violet®, Chroma) solution.

2. Immunohistochemistry

A total of twelve animals were used for the IHC, and divided into three groups of four animals. Using an enzyme-labeled polymer method (EnVision+ system®, DAKO), IHC was employed on the sections obtained from the animals in these three groups to analyze the detailed distribution of the GFAP, SP and CGRP, respectively. The sections were incubated in a mouse monoclonal anti-GFAP (dilution 1:1000, SIGMA), a rabbit polyclonal anti-SP (dilution 1:3000, CHEMICON) or a rabbit polyclonal anti-CGRP (dilution 1:3000, Amersham) serum diluted in a 0.1 M PBS containing 0.1% Triton X-100 overnight at 4°C. After rinsing, they were incubated with a secondary antibody (Labeled polymer, HRP®, DAKO) for 1 h at room temperature. Next, they were exposed to a 50 mM Tris-HCl buffer containing 0.01% 3-3'-diaminobenzidine 4HCl (DAB) and 0.005% H₂O₂.

3. Double-labeling immunofluorescence

To investigate a morphological interrelationship between the astrocytes and the nerve endings, a total of eight animals were used for double-labeling

IF, and divided into two GFAP/SP- and GFAP/CGRP-groups of four animals.

The sections were incubated with (1) GFAP/SP (dilution GFAP 1:1000; SP, 1:3000) or (2) GFAP/CGRP (dilution GFAP 1:1000; CGRP, 1:3000) serum diluted in a 0.1 M PBS containing 0.1% Triton X-100 overnight at 4°C. And they were incubated with a 0.1 M PBS containing fluorescein-isothiocyanate (FITC)-labeled anti-mouse IgG [FLORESCIEIN ANTI-MOUSE IgG(H+L)®, dilution 1:1000, VECTOR] and tetramethylrhodamine-isothiocyanate (TRITC)-labeled anti-rabbit IgG (Dky X Rbt IgG RHODAMINE®, dilution 1:1000, CHEMICON) for 4 h at room temperature. After the immuno-reaction, the Nissl staining was performed in the same section to determine the expression lamina of the GFAP/CGRP- or GFAP/SP-positive reactions in the Sp5C.

The sections that underwent the Nissl staining and the IHC were examined with a light microscope (MICROPHOT-FXA®, Nikon) and those with the double-labeling IF with a fluorescence microscope (BX50®, OLYMPUS).

B. Electron Microscopic Investigation

For the electron microscopic investigation, a total of eight animals were used for double-labeling EMIHC, and divided into two GFAP/SP- and GFAP/CGRP-groups of four animals. In the present study, GFAP-positive reactions were visualized by DAB with silver-gold intensification (DAB-SGI), and SP- or CGRP-positive reactions were detected by DAB alone.

After the perfusion with a fixative containing 4% paraformaldehyde and 0.35% glutaraldehyde in 0.1 M PB (pH7.4), the tissue blocks were post-fixed in a fixative containing 4% paraformaldehyde in 0.1 M PB (pH7.4) for 24 h at 4°C. Serial frontal sections (40 µm in thickness) were cut with a microlicer (VIBRATOME Series 3000 SECTIONING SYSTEM®, TECHNICAL PRODUCTS INTERNATIONAL INC.). The sections were first incubated with the anti-GFAP serum as previously mentioned, and then processed for the DAB-SGI according to the method reported by Liposits *et al.*¹¹⁾ In the next step, the sections were further incubated with the anti-SP or the anti-CGRP serum for 72 h, and treated with a avidin-biotin complex (ABC) method.

After the immuno-reaction, the sections were fixed in chilled 1% OsO₄ solution for 1 h at 4°C, dehydrated through a graded series of acetone and embedded in Epon812 mixture (LUVAK®, nacalai tesque). Ultrathin sections were cut with a ultramicrotome (LEICA ULTRA UCT®, Nissei Sangyo co Ltd.), stained with uranyl acetate, and

examined with a transmission electron microscope (H-7100®, HITACHI).

The specificity of the anti-GFAP, the anti-SP and the anti-CGRP serum was confirmed by the pre-absorption with synthetic peptide respectively. We also confirmed that cross-reaction in the double-labeling IHC did not occurred using negative control serum (Universal Negative Control-Mouse, Rabbit®, DAKO).

Results

1. Nissl staining

The location of the Sp5C could be identified by the Nissl staining (Fig. 1). In the higher magnification view, four laminae were observed in the Sp5C (Fig. 3B, 3D). However, the borderline between lamina III and lamina IV was not obvious (Fig. 3B, 3D).

2. Immunohistochemistry

(1) GFAP

GFAP-positive reactions were observed throughout all of the Sp5C lamina, and especially in lamina I and II (Fig. 2A, 2D).

(2) SP and CGRP

SP- or CGRP-positive reactions were demonstrated only in lamina I and II of the Sp5C. SP- or

CGRP-positive nerve endings were identified as small dots, and a large number of CGRP-positive nerve endings were observed, in comparison with the SP-positive nerve endings (Fig. 2B, 2C, 2E, 2F).

3. Double-labeling immunofluorescence

FITC-labeled astrocytic cell bodies and processes (green-colored) were showed a cobweb-like shape with processes extending in all directions (Fig. 3A, 3C). According to the simultaneous visualization of the IF and the Nissl staining (Fig. 3B, 3D) in the same section, we observed GFAP-positive astrocytes (FITC-labeled; green-colored) and SP- or CGRP-positive nerve endings (TRITC-labeled; red-colored) in lamina I and II (Fig. 3A, 3C).

4. Double-labeling electron microscopic immunohistochemistry

In the lamina I and II of the Sp5C, many GFAP-positive processes with DAB-SGI were observed. And theses astrocytic processes made the direct contacts with the SP- or CGRP-positive nerve endings with DAB alone (Fig. 4A, 4B).

Discussion

As shown in Fig. 1, we identified four laminae of the Sp5C according to the existence of the large neurons⁵⁾. However, the border line between the lamina III and IV was not obvious⁵⁾. Based on these laminar structures, the distribution of the astrocytes in the Sp5C and the morphological interrelationship between the astrocytes and nerve endings were discussed.

1. Distribution of the astrocytes in the Sp5C

Our IHC results clarified that many astrocytes were located in lamina I and II, which received nociceptive information from oro-facial regions. Additionally, other studies have shown the presence of SP- or CGRP-positive nerve endings in lamina I and II of the Sp5C^{12,13)}, and our study obtained the same results. Therefore, it was apparent that astrocytes were distributed in the region, which contained many SP- or CGRP-positive nerve endings.

2. Morphological interrelationship between astrocytes and nerve endings

We have previously demonstrated the presence of astrocytic contacts with other nerve endings in the other regions of the brain^{14,15)}. In the present study, using the double-labeling IF and EMIHC, dense SP- or CGRP-positive reactions were observed around the GFAP-positive reactions, and we

Fig. 1. Nissl staining section of the Sp5C. The location of the Sp5C was identified in the Nissl-stained section.

*: spinal trigeminal tract, broken line: Sp5C

Fig. 2. Immunohistochemistry for GFAP, SP and CGRP. Positive reactions for GFAP (2A), SP (2B) or CGRP (2C) were observed in the Sp5C. Fig. 2D, 2E and 2F indicate a higher magnification view of square regions in Fig. 2A, 2B and C, respectively. GFAP-positive reactions were observed in all laminae of the Sp5C, and SP- or CGRP-positive nerve endings were observed in the lamina I and II.

also clarified the morphological contact between astrocytic processes and SP- or CGRP-positive nerve endings in the lamina I and II of the Sp5C.

It has also been reported that astrocytes, which support and nourish the neurons, also regulate extra-cellular ion concentrations and release some substances into the extra-cellular space in response to various kinds of neurotransmission^{16–20}. Additionally, it has been demonstrated that some physiological stimuli such as aging and the light-dark conditions cause morphological and functional changes to astrocytes^{21,22}.

In addition, the following findings have been reported: 1. Astrocytes are activated within the thoracic spinal cord following the intramyocardial injection of formalin⁸), 2. In spinal astrocytes, pain stimulation to the nerve endings induces changes in some kinds of substances related to the cellular signal transduction²³), 3. In the process of the nociceptive information transmission, SP in the nerve endings are related to the synthesis of the prostaglandins in the astrocytes²⁴), and 4. SP- and CGRP-receptors exist in the astrocytes^{25,26}). Therefore, our morphological results suggested that astrocytes

Fig. 3. Double-labeling immunofluorescence. Four laminae were identified in the Sp5C (3B, 3D). GFAP/SP (3A)-, or GFAP/CGRP (3C)-positive reactions were observed in lamina I and II in the Nissl staining sections of the Sp5C (3B, 3D). I, II, III, IV: lamina I–IV (from the outside lamina I to the inside lamina IV, sequentially), *: spinal trigeminal tract

in the Sp5C might receive the nociceptive information from the primary afferent nerve endings, and also that they might play some important roles in the transmission of the nociceptive information from the oro-facial regions.

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Fig. 4. Double-labeling electron microscopic immunohistochemistry. In the lamina I and II, GFAP-positive astrocytic processes with DAB-SGI were observed to make direct contacts with SP(4A)- or CGRP(4B)-positive nerve endings with DAB alone. DAB: 3-3'-diaminobenzidine 4HCl, DAB-SGI: DAB with silver-gold intensification

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