The localization of calcitonin gene-related peptide in the human trigeminal ganglion and masseter muscle

By

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Summary: The localization of calcitonin gene-related peptide (CGRP) is similar to that of a neurotransmitter which indicates masticatory muscle pain in the area of the masseter fascia. CGRP is released from the trigeminal ganglion (TG). The aim of this study was to analyze the distribution of CGRP in the fascia of the masseter muscle (FMM) and TG in a morphometric manner, with respect to the location and density of CGRP-immunopositive reaction fiber (CGRP-IRF). A higher number of the CGRP-IRF were mainly found located around elongated blood vessels and small nerves on the origin side of the middle zone FMM in the O group (presented with occlusion). In the sectional histochemical analysis of the O group, the CGRP-IRF were clearly detected in oval vessels, large elongated vessels and large nerves in contrast with that of the Non-O group (presented with no occlusion) samples. The number of CGRP-immunopositive ganglion cells (CGRP-IPGCs) in the O group mandibular nerve division was higher than that of other divisions. A reduction of the CGRP-IRF numbers were found in the no-loading groups. The characterization of these locations of CGRP-IPGCs can also provide useful data for the understanding of myofascial pain syndrome of the masseter muscle (MM).

Introduction

Myofascial pain syndrome (MPS) is related to a tenderness point (tender points) or trigger point present in the muscle, tendon, or ligament of the TMJ. The immobility of the fascia collagen affects C fibers excited by vascular systems. In our previous studies, we found that calcitonin gene-related peptide (CGRP) was localized near nociceptive receptors and that CGRP affected the vessels on the surface of the maxillary sinus mucosa (Sato et al., 2012). We also found that CGRP affected pain and hearing through its actions in the human tensor tympani muscle (Yamazaki and Sato, 2014). During mouse masseter muscle (MM) development, CGRP may have an influence on embryonic myosin heavy chain. CGRP also affects the angiogenesis markers at embryonic stages (Azuma et al., 2016). The administration of a central muscle relaxant and stellate ganglion block (SGB) are performed for chronic pain avoidance by sympathetic tone, but these treatments have strong side effects. Therefore, there is a need to treat only the site of pain in a localized manner for the treatment of MPS, and thus, we need to examine the localization of CGRP in a similar manner to that of a neurotransmitter, which for MPS, indicates the same region as pain nerves in the masseter fascia that are active during temporomandibular disorder. Masticatory muscle pain is limited compared to normal cases (Lund et al., 1991). The presence of a tender point indicates the relevance of the muscle nodule (Mense et al., 2003). Previous reports have indicated a relationship among tender points and pain, muscle induration, endplate (Hubbard and Berkoff, 1993) in MPS. In the mouse gluteus maximus muscle, enhanced sympathetic nerve activity and endothelial dysfunction occurs during aging. However, the reactivity among branches of microvascular resistance networks were adapted to the aging of muscle (Sinkler and Segal, 2014). Muscle sympathetic nerve activity is reduced by the leg resistance vasculature of the conduit arteries in older humans (Parker et al., 2007). In general, decreases in neuropeptide synthesis, content and release contribute to the decrease in neurogenic inflammation in old rats (Khalil et al.,...
However, these mechanisms have not yet been elucidation remained unchanged (Warburton and Santer, 1994). CGRP-immunopositive reaction of the pelvic visceral sensory innervation pattern and density of CGRP-immunopositive reaction was studied in two rat strains in Glenn and Duckles (1994). In contrast, the NPY response from 12 to 24 months in the study of two muscle (Hodges et al., 2009); there was no neuronal changes with age in muscle (Hodges et al., 2009). Muscle sympathetic nerve activity is influenced by gravitational stress and aging (Mano, 2001). Neuropeptide Y (NPY) is an important sympathetic neurotransmitter involved in neurovascular regulation. The NPY system also declines with age in muscle (Hodges et al., 2009); there was no NPY response from 12 to 24 months in the study of two rat strains in Glenn and Duckles (1994). In contrast, the pattern and density of CGRP-immunopositive reaction fiber (CGRP-IRF) of the pelvic visceral sensory innervation remained unchanged (Warburton and Santer, 1994). However, these mechanisms have not yet been elucidated clearly. Therefore, the present study used immunohistochemical analysis of whole-mount specimens and sections to examine the distribution of CGRP in the human MM during MPS.

### Materials and methods

#### Materials

In this study, the fascia of masseter muscle (FMM) and trigeminal ganglion (TG) of 22 human cadavers ranging from 65- to 104-years old (mean 82.1 ± 12.1 years old; male, n = 12, 80.1 ± 9.1; female, n = 10, 84.6 ± 15.1 years old) were examined. We classified them into the following two groups: the O group (male, n = 6, female, n = 5), which presented with occlusion, and the Non-O group (male, n = 6, female, n = 5), which presented with no occlusion (occlusion was not sufficient; edentulous, no opposing teeth or partial denture specimens). All human cadavers used in the study had been donated for dissection. The samples were injected with 10% formalin with return perfusion via the femoral artery. After anatomical dissection, the muscle fascia was removed from the MM. The samples were then used for immunohistochemical analysis of the FMM.

#### Immunohistochemical methods

**Whole-mount staining methods**

FMM removed from the cadavers was fixed again for 2–3 days with 10% neutral formalin and stored in 70% EtOH. The samples (n = 10) were incubated for 2 h at room temperature with 100% EtOH; this step was repeated two times and then incubated overnight with 100% EtOH. After exchanging the liquid for a mixture of a 1:2 volumetric ratio of MeOH: chloroform, samples were incubated overnight at 2–8°C under agitation. The samples were then incubated for 4 h at room temperature with 100% MeOH, incubated for 4 h at room temperature with 100% EtOH, and incubated overnight at 2–8°C with 100% EtOH. Fat was then removed from the samples. Whole-mount samples were washed with phosphate buffered saline (PBS) and incubated with 3% H₂O₂ for 1 h to eliminate endogenous peroxidase activity. The samples were then washed with PBS. After 2 overnight incubations at 4°C in PBS containing 2% Triton X-100, the samples were washed again with PBS and incubated for 2.5 h at room temperature with 2% normal goat serum/PBS containing 0.05% Tween 20 to prevent non-specific antibody binding. Right fascia samples were then incubated for 4 days at 4°C with primary rabbit polyclonal antibodies against CGRP (1:1,000; Enzo Life Sciences, USA), Substance P (SP) (1:1,000; Enzo Life Sciences, USA), Protein gene product 9.5 (PGP9.5) (1:1,000; UltraClone Ltd., UK) or with normal goat serum as the negative control (left fascia samples). The samples were then washed with PBS containing 0.05% Tween 20 and incubated with secondary antibody of horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200, Santa Cruz Biotechnology, USA) according to the manufacturer’s instructions. The samples were then washed with PBS. The staining was visualized using diaminobenzidine. Appropriate negative controls were included. Images were acquired using a stereomicroscope (Leica MZ 16; Leica Microsystems, USA) with the Leica Application Suite software (Leica Microsystems, USA). We also define immunopositive localization on the vessels and

| Table 1. CGRP-immunopositive reaction levels and distribution in whole-mount staining of FMM. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | anterior zone   | middle zone     | posterior zone  | anterior zone   | middle zone     | posterior zone  |
|                                | inner surface   |                 |                 | outer surface   |                 |                 |
| O group                         |                 |                 |                  |                 |
| origin side                     | +               | +++             | +               | ±               | ±               | ++             |
| intermediate region             | –               | +               | –               | ±               | ±               | +              |
| insertion side                  | –               | –               | ++              | ±               | ±               | ±              |
| Non-O group                     |                 |                 |                  |                 |
| origin side                     | –               | –               | ++              | –               | –               | –              |
| intermediate region             | –               | –               | –               | –               | +               | –              |
| insertion side                  | –               | –               | –               | –               | –               | –              |

The immunopositive localization on the vessels and nerves were classified five morphological reaction structure such as follows: (+++) strong concentration of mesh-like structure, (++), moderate concentration of mesh-like structure, (+) weak concentration of fiber-like structure, (±) very low concentration of fine fiber-like structure, (-), no or very low concentration reaction of fine fiber-like structure.
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nerves which classified five morphological reaction structure such as follows: (+++) strong concentration of mesh-like structure, (++), moderate concentration of mesh-like structure, (+) weak concentration of fiber-like structure, (±) very low concentration of fine fiber-like structure, (-), no or very low concentration reaction of fine fiber-like structure in 9 zone of each human FMM (see Table.1, Fig. 1).

Paraffin-embedded sections of CGRP

FMM and TG were fixed with Tissue Fixative (Genostaff Co., Ltd, Tokyo, Japan), embedded in paraffin and sectioned at 5 µm. The tissue sections of FMM (cross-section of origin side of the middle zone) and TG (sagittal section) were deparaaffinized with xylene and rehydrated through a series of ethanol solutions in PBS. Endogenous peroxidase was blocked with 0.3% H₂O₂ in methanol for 30 min, followed by incubation with Protein Block (Genostaff Co., Ltd.) and an avidin/biotin blocking kit (Vector SP-2001) (n = 6). The sections were then incubated with rabbit polyclonal antibodies against CGRP (10 µg/ml; ENZO BML-CA1134) and normal rabbit Ig as a negative control for CGRP at 4°C overnight. These sections were washed with TBST and TBS. They were incubated with biotin-conjugated goat anti-rabbit IgG (Dako E0432) at room temperature for 30 min. After washing with TBST and TBS, they were incubated with peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan) at room temperature for 5 min. Peroxidase activity was visualized by diaminobenzidine. The sections were

![Fig. 1. Schema of 9 immunopositive observations of inner and outer surface in human FMM. O = origin side; IR = intermediate region; I = insertion side; A = anterior zone; M = middle zone; P = posterior zone.](image-url)
counterstained with Mayer’s hematoxylin (MUTO Pure Chemicals Co., LTD.), dehydrated, and then mounted with Malinol (MUTO Pure Chemicals Co., LTD.). Images were acquired using a microscope (Leica DM 2500 16; Leica Microsystems, USA) and Leica Application Suite software (Leica Microsystems, USA). We also measured the number of positive cells of the TG in each section by using random sampling methods on each small area (0.25 mm², n = 90).

Statistical analysis
The measurement data of CGRP-immunopositive ganglion cells (CGRP-IPGCs) in TG were assessed using the Pearson’s chi-square test to test the validity of a distribution assumed for occlusion loss. Results were expressed %. The level of significance (p) was calculated at Person’s chi-square test (p < 0.001). The statistical analyses were performed using the IBM SPSS Statistics Base, version 22 (IBM, Chicago, USA).

Ethics
The study was approved by the Human Research Committee of Nippon Dental University (no. NDU-T2015-20). The human cadavers were obtained from a donor-based system using the guidelines included with the Law Concerning Body Donation for Medical and Dental Education (the Body Donation Law).

Results

Whole-mount CGRP immunohistochemical staining of FMM

O group
The simple branch of CGRP-IRF and CGRP-immunopositive mesh-like structure were also found in the inner and outer surface of the FMM. A substantial number of CGRP-IRF were detected on small blood vessels and nerve fibers on the origin side of the posterior inner FMM. A few scattered CGRP-IRF were visualized in the intermediate region of the middle zone of the outer surface of FMM. Very low reaction of CGRP-IRF were scattered in other regions of the FMM (Figs. 1, 2) (table 1).

Non-O group
The simple branch of CGRP-IRF and CGRP-immunopositive mesh-like structure were also found in the inner and outer surface of the FMM. A substantial number of CGRP-IRF were detected on small blood vessels and nerve fibers on the origin side of the posterior inner FMM. A few scattered CGRP-IRF were visualized in the intermediate region of the middle zone of the outer surface of FMM. Very low reaction of CGRP-IRF were scattered in other regions of the FMM (Figs. 1, 2) (table 1).

Whole-mount SP and PGP9.5 immunohistochemical staining of FMM
SP-IRF were mainly detected on the small blood vessels. PGP9.5-IRF were also located around large blood vessels and on small nerves. (Fig. 3)

CGRP immunoreactivity of serial sections of FMM in O group and Non-O group
In the sectional histochemical analysis of the O group FMM, in the subcutaneous tissue layer, the CGRP-IRF were clearly visible on oval vessels and large elongated vessels and large nerves. This result was in contrast with the Non-O group samples in which CGRP-IRF were slender and found on very small vessels and small nerves. In the fat layer beneath the connective tissue layer, CGRP-IRF were present on numerous oval vessels and scattered in O group samples compared to Non-O group samples on very small vessels surrounded by fat cells. In the FMM, above the tendon layer, CGRP-IRF on very small oval vessels were scattered in O group samples, compared with Non-O group samples that had a small concentration of CGRP-IRF observed on very small vessels. Moreover, in the FMM layer beneath the tendon layer, the presence of CGRP-IRF on large oval and elongated vessels were found in O group samples, in contrast with Non-O group samples that had a very low amount of CGRP immunoreaction. (Figs. 4, 5).

CGRP immunoreaction in TG in O group and Non-O group
In sagittal sectional histochemical observations of the TG, the CGRP-IPGCs were found on three divisions (ophthalmic, maxillary, and mandibular nerves) of the TG in both groups. Non-O group samples that had CGRP-immunopositive oval cells were scattered throughout the three divisions of the trigeminal ganglion. In the ophthalmic and mandibular nerve divisions, CGRP-IPGCs were more abundant in the O group than the Non-O group. In the maxillary nerve division of the Non-O group, the proportion of CGRP-IPGCs were higher than that of the O group (p < 0.001). In the mandibular nerve division, CGRP-IPGCs were also more abundant in the O group than the Non-O group (p < 0.01). In the Non-O group, specific intercellular spaces could be identified mainly found comparison with the O group (Figs. 6, 7) (Table 2).
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Data analysis of CGRP-IPGCs in TG

The percentage of CGRP-IPGCs of the mandibular division in the O group was higher than that of the ophthalmic and maxillary divisions, in contrast with that of the maxillary division compared to ophthalmic and mandibular divisions in the Non-O group. Therefore,

Fig. 2. CGRP immunoreaction of the blood vessels and small nerves in the O group and Non-O group in FMM by whole-mount staining samples at the macroscopic level. Positive signals for CGRP antibody are indicated by arrowheads in A–F. A. The origin side in the O group. The reaction of simple branch of CGRP-IRF. B. The origin side in the Non-O group. The reaction of CGRP-immunopositive mesh-like structure. C. The middle zone of the inner surface in the O group. The reaction of CGRP-immunopositive small mesh-like structure. D. The middle zone of the inner surface in the Non-O group. The reaction of CGRP-immunopositive small mesh-like structure. E. The insertion side region of the posterior inner surface in the O group. The reaction of simple branch of CGRP-IRF. F. The insertion side region of the posterior inner surface in the Non-O group. The reaction of simple branch of CGRP-IRF. O group (Male, 77-years old), Non-O group (Male, 90-years old); Bar = 300 µm
the number of CGRP-IPGCs was affected by the loss of occlusion. In our chi-square test analysis, the percentage of the positive cells among the maxillary, mandibular and ophthalmic divisions was significantly influenced by occlusion loss ($\chi^2 = 240.27$, df = 2, $p < 0.001$) (Table 2).

**Discussion**

**CGRP in the FMM**

Interactions among the peripheral nervous system, the immune system, and local cells are probably of great importance for the modulation of pain and inflammation in the TMJ and orofacial musculature (Kopp, 2001). Simultaneous neurogenic release of CGRP in human skeletal muscle may induce myofascial pain (Pedersen-Bjergaard et al., 1991) was indicative of shoulder pain through the distribution of CGRP using immunohistochemical methods (Yoshida et al., 1995). Therefore, the localization of neural factors with CGRP-IRF reflects pain in the human FMM. In our immunohistochemical observations, CGRP-IRF formed mesh-like structure were located in the middle region of the origin of the MM as shown in whole-mount immunohistochemical staining specimens. Moreover, CGRP-immunopositive reactional regions were also mainly localized in the subcutaneous tissue layer, fat layer and FMM layer beneath the tendon layer of human MM in the serial sections of the MM and FMM. These reaction sites indicate pain or inflammatory zones, such as tender points during movements of the MM. These reaction sites of CGRP antibodies are markers for contraction and expansion of pain and vasodilation, especially for the FMM middle zone of the origin of the MM that indicates pain sensitivity compared to other sites, and it may suggest the presence of a tender point of the human MM. In general, CGRP is one of several important mediators for facial inflammation (Multon et al., 2005), TMJ pain (Haechi et al., 1999; Sato et al., 2004) and migraine (Benemei et al., 2009 a, b). CGRP is released from TG afferent nerve fibers during migraine and causes neurogenic inflammation (Russell et al., 2014). CGRP is released from the ipsilateral TG (Ambalavanar et al., 2006). The localization of CGRP mainly migrated from TG in the MM. The CGRP-IRF were mainly detected in close proximity to muscle bundles and the blood vessels (Carleson et al., 2004). CGRP is a potent vasodilator of peripheral blood vessels (Ohlén et al., 1987). CGRP mainly has an important role in activating vasorelaxation at local levels (Russel et al., 2014). CGRP-IRF were found scattered around the capillary. CGRP-IRF was concentrated in the middle zone of the origin where many blood vessels are admitted by vascular markers in our results. In addition, the blood vessel was mainly distributed in the subcutaneous layer and the FMM. The afferent nerve of stimulation as a sensory fiber was mainly found in the middle

![Fig. 3. SP and PGP9.5 immunoreaction of the blood vessels and small nerves in FMM by whole-mount staining samples at the macroscopic level. Positive signals for SP and PGP9.5 antibody are indicated by arrowheads in A, B, A. Positive signals for SP in the O group (Male, 92-years old). B. Positive signals for PGP9.5 in the O group (Female, 93-years old). C. Negative control of Fig. 3B. (Female, 93-years old). Bar = 300 µm](image-url)
Fig. 4. CGRP immunoreaction of serial sections from the O group in FMM. Positive signals are indicated by arrowheads in A–H. A. Immunohistochemical localization of anti-CGRP antibody in the subcutaneous tissue layer of the cross-section. B. Negative control of Fig. 4A. C. CGRP-immunopositive reactions were found in the blood vessels in the fat layer. D. Negative control of Fig. 4C. E. CGRP-immunopositive reactions were also found in the very small vessels above the tendon layer. F. Negative control of Figs. 4E. G. CGRP-immunopositive reaction were clearly found on large oval vessels beneath the tendon layer. H. Negative control of Fig. 4G. Male, 80-years old; BV, blood vessel; CT, connective tissue; PN, peripheral nerve; Bar = 30 μm.
Fig. 5. CGRP immunoreaction of serial sections of the Non-O group in FMM. Positive signals are indicated by arrowheads in A-H. A. CGRP-immunopositive reactions were found in the subcutaneous tissue layer of the cross-section in large blood vessels. B. Negative control of Fig. 5A. C. Weak CGRP-immunopositive reactions were found in the blood vessels in the fat layer. D. Negative control of Fig. 5C. E. Very weak CGRP-immunopositive reactions were also found on small blood vessels above the tendon layer. F. Negative control of Fig. 5E. G. CGRP-immunopositive reaction were clearly found on large oval vessels beneath the tendon layer. H. Negative control of Fig. 5G. Male, 88-years old; BV, blood vessel; CT, connective tissue; Bar = 30 µm.
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In general, muscles experience reduction of tension, functional strength, prolongation and contraction of MM.
during aging (Larsson, 1978). The cross-sectional area and density of mass of MM also indicated decreased levels during aging (Newton et al., 1987). Muscle activity levels were also affected by edentulous or denture-treated subjects (Kapur et al., 1984; Salonen et al., 1994; Raustia et al., 1996). Mechanical stress applied
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Table 2. Analysis data of CGRP-IPGCs in TG.

<table>
<thead>
<tr>
<th>Ophthalmic nerve</th>
<th>Maxillary nerve</th>
<th>Mandibular nerve</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>O group</td>
<td>Non-O group</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>17.5%</td>
<td>9.6%</td>
<td>27.1%</td>
<td></td>
</tr>
<tr>
<td>13.0%</td>
<td>19.6%</td>
<td>32.6%</td>
<td></td>
</tr>
<tr>
<td>25.5%</td>
<td>14.7%</td>
<td>40.2%</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>56.0%</td>
<td>44.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

Results are expressed%. P is calculated at Person’s chi-square test (p < 0.001).

to the rat temporomandibular joint increased opening duty time of the MM, whereas the stress of the digastric decreased activity level significantly (Kawai et al., 2008). We conducted immunohistochemical staining of CGRP between occlusion (O group) and specimens with occlusion, which was not sufficient with edentulous or no opposing teeth (Non-O group). These groups exhibited differential patterns of CGRP-immunopositive reaction levels such as a mesh-like structure in our results. The sensory nerves in the masseter area may be controlled by maxillary and mandibular divisions. In our results, the proportion of CGRP-IPGCs indicated different compositions between maxillary nerve and mandibular divisions between the O group and Non-O group. The proportion of CGRP-IPGCs of the mandibular division in the O group was larger than that of Non-O group (p < 0.001). After loss of teeth, the mastication stress of occlusion may be mainly controlled by the mandibular nerve division. The reduced CGRP-IPGCs of the mandibular division were found in the Non-O group, where occlusion was not sufficient for edentulous or no opposing teeth in the mandible. In contrast, the O group indicated increased occlusal loading, which reflected that muscle sympathetic nerve activity increased at the masseter resistance vasculature in humans in the mandible. Efforts are focused on determining a CGRP antibody that specifically identifies active vasculature in the maxilla and mandible of human subjects. In our results, differences of CGRP-immunopositive reaction levels were found between no-loading and loading subjects. The strong activity such as a mesh-like structure was mainly found in the blood vessels with CGRP-IRF on the origin side of the middle zone of the O groups in the dentulous subjects. Moreover, CGRP-IRF were also found in the TG of the O and Non-O groups, and especially strong activity was detected in the TG of the O group compared to that of Non-O group subjects. According to the different reaction of CGRP-immunopositive reaction levels found between O group and Non-O group subjects, the CGRP of the FMM is mainly released from TG. In general, orofacial pain is a modulator of trigeminal system functions. The jaw muscle pain has several important effects on the sensory and motor function of the mastication with functional levels of the trigeminal system (Lobbezoo et al., 2002). However, there are two ways to reduce CGRP-IPGCs to aging or no-loading in human MM. These two ways effect the MM specific activity. Therefore, we tried to investigate the effects of aging between subjects in the O group and Non-O group. We concluded that reduction of CGRP-IRF were found in the no-loading groups compared to that of occlusal subjects.

Conflicts of interest

We certify that there are no conflicts of interest with any financial organization regarding the material discussed in the manuscript.

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Reference


