Muscle and Brain Changes of Calcitonin Gene-Related Peptide in Experimentally Induced Unilateral Rat Masseter Myositis

Joakim Carleson, DDS, PhD
Departments of Physiology and Pharmacology, Clinical Oral Physiology, and Surgery and Rehabilitation

Thomas Lundeberg, MD, PhD
Professor
Department of Physiology and Pharmacology and Department of Surgery and Rehabilitation

Björn Appelgren, DDS, MA, PhD
Associate Professor
Department of Physiology and Pharmacology and Department of Clinical Oral Physiology

Karolinska Institutet
Stockholm, Sweden

Correspondence to:
Dr Joakim Carleson
44 Museum Road
Torquay TQ1 1DW
England
Fax: + 44 1803 380045
E-mail: info@monzan.com

Aims: *To investigate changes in calcitonin gene-related peptide (CGRP)–like immunoreactivity (CGRP-LI) in the rat masseter muscle and brain after the unilateral experimental induction of masseter myositis.* ***Methods:*** *Ipsilateral and contralateral changes of the CGRP were examined in rat masseter muscle after the induction of unilateral myositis on the right side with an intramuscular injection of 0.01 mL Freund's adjuvant. The left masseter, and left and right masseters of control rats, were injected with 0.01 mL saline (0.9%). After 21 days, tissue samples from the masseter muscles and the hypothalamic-pituitary-adrenal (HPA) axis were analyzed for the presence of CGRP by immunohistochemistry, radioactive immunoassay, and high performance liquid chromatography. Hematoxylin-eosin staining was used to confirm inflammation in the masseter muscles.* ***Results:*** *Elevated CGRP-LI was detected bilaterally in the masseter muscles (*$P < .001$*) in the myositis group. CGRP-immunoreactive nerve fibers were mainly detected in close proximity to muscle cells and in the walls of the blood vessels. Compared to the control rats, a significant difference in scratching behavior was seen in the myositis group from day 9 until day 21. In the myositis group, CGRP-LI was increased in the pituitary gland concomitant with the increase in CGRP-LI in the masseter muscles but was decreased in the hypothalamus. A possible explanation for these changes could be that rats with chronic myositis develop an abnormal function of the HPA axis triggered by masseter muscle inflammation.* ***Conclusion:*** *The results of this study demonstrate that CGRP may play an important role both peripherally and centrally in masseter muscle myositis in association with presumed nociceptive behavior.* J OROFAC PAIN 2004;18:246–252

Key words: calcitonin gene-related peptide, Freund's adjuvant, inflammation, masseter muscles, myositis, neurogenic inflammation, neuropeptides, scratching

The nervous system plays a significant role in the inflammatory process and is involved in the release of sensory neuropeptides.[1] Recently Reinert et al[2] investigated the density of substance P–immunoreactive nerve endings in normal and inflamed rat muscle. In muscles with inflammation induced by an injection of Freund's adjuvant they found increased density of substance P and increased axonal sprouting, regeneration, and synaptic reorganization.[1,3] Calcitonin gene-related peptide (CGRP) is another peptide with a suggested role in the inflammation. It has been demonstrated to modulate edema formation[4,5] and may influence nociceptive transmission at the spinal cord and more central

levels.[5,6] Recently, our research team showed in rats that a peripheral inflammatory process in the temporomandibular joint results in changes of CGRP concentrations in the central nervous system.[7,8] CGRP stimulates the hypothalamo-pituitary-adrenal (HPA) axis after intracerebroventricular injection in rats[9] and may be important in regulating pituitary cell function.[10] Via the portal system, hypothalamic hormones regulate the anterior pituitary by CGRP-containing nerve fibers in the anterior pituitary.[11] CGRP-immunoreactive fibers exist in the anterior, intermediate, and posterior lobes as well.[12] During inflammation and nociception, the HPA axis has increased activity, and the levels of adrenocorticotrophic hormone increase as a result of the stimulatory effect of CGRP.[13] The aim of this study was to investigate changes in CGRP-like immunoreactivity (CGRP-LI) in the rat masseter muscle and brain after the unilateral experimental induction of masseter myositis by injection of Freund's adjuvant. In order to determine a possible relationship between the concentrations of CGRP-LI and presumed nociceptive behavior in masseter myositis, the rats were also examined for back-paw scratching behavior.

Materials and Methods

These experiments were performed according to a protocol that was approved by the Karolinska Institute ethical committee. Twenty male albino Sprague-Dawley rats (200 to 250 g) were habituated to the laboratory for at least 7 days before experimentation. All rats were maintained under identical conditions, ie, alternate cycles of 12 hours of light and 12 hours of darkness, temperature of 24°C, 60% relative humidity, and food and water ad libitum. The rats were anesthetized with chloral hydrate (0.4 g/kg), the skin overlying the masseter muscles was shaved, and an intramuscular injection was carried out with a 27-gauge needle. The rats were grouped as follows: 10 were inoculated intramuscularly with 0.01 mL heat-killed Mycobacterium butyricum (10 mg/mL, H37 RA, nonviable, desiccated; Difco Lab) in paraffin oil (Difco Lab), ie, Freund's adjuvant, in the right masseter muscle and injected with 0.01 mL saline (0.9%) in the left masseter muscle. Ten rats were injected with 0.01 mL saline in the left and right masseter muscles.

Scratching behavior was defined as the animal touching the skin overlying the masseter muscle with the back paw. To obtain a baseline of the normal scratching behavior, the rats were surveyed on the seventh day before inoculation/injection, the third day before inoculation/injection and the day before the inoculation/injection. They were then observed on every third day for 21 days. The rats were observed in individual cages and the total time of scratching behavior was recorded during 30-minute periods. One hour of acclimatization was given before the evaluation with the cages placed 1 m in front of the observer in a quiet room.

Each time the rats were monitored for scratching behavior, they were also monitored for clinical signs of inflammation, such as changes in skin temperature, swelling, and color. Skin temperature was monitored indirectly with a laser-sensitive device (Bio-Medical Instruments).

The rats were anesthetized and killed by decapitation 21 days after inoculation/injection. Samples of blood plasma were taken. The hippocampus, hypothalamus, pituitary, and masseter muscles were extirpated and immediately frozen on dry ice. For the immunohistochemistry analysis, an additional 3

10 minutes. They were then washed in running tap water for 10 minutes; counterstained with eosin for 30 seconds; and finally dehydrated in 70%, 95%, and absolute alcohols (2 changes of 2 minutes each or until excess eosin was removed). A Nikon epifluorescence microscope was used to analyze the sections for CGRP-LI. T-Max black-and-white film (Kodak) was used to photograph the sections and other aspects of the study.

Prior to extraction, the tissues were cut into small pieces, boiled for 10 minutes in 1 mol/L acetic acid in 4% ethylenediaminetetraacetic acid (EDTA), homogenized in a Polytron (15 seconds), sonicated (30 seconds), and centrifuged at 3,000 g for 15 minutes. The supernatants were lyophilized and diluted in 2-mL PBS containing 0.2% bovine serum albumin and 0.1% Triton X-100 (Amarsham Biosciences). These samples were kept at –70°C until analysis with competitive radioimmunoassay. Blood (1.5 to 4.5 mL) was collected with a Vacutainer tube containing 143 IU heparin (LEO Pharma), and Trasylol (Bayer) was added. The samples were centrifuged and plasma was removed and frozen at –70°C. Blood-plasma samples were extracted using a reverse-phase C18 cartridge (Sep-Pak).

The CGRP-LI was analyzed using antiserum CGRPR8 raised against conjugated rat CGRP. Reverse-phase high-performance liquid chromatography (HPLC)–purified 125 I-Histidyl rat CGRP was used as a radioligand and rat CGRP as the standard. The cross-reactivity of the assay to substance P, neurokinin A, neurokinin B, neuropeptide K, gastrin, neurotensin, bombesin, neuropeptide Y, and calcitonin was less than 0.01%. Cross-reactivities toward rat CGRP α and β were 100% and 120%, respectively. Intra- and interassay coefficients of variation were 8% and 14%, respectively. A protein analysis,[14] slightly modified, was carried out in order to determine the amount of protein in the samples in relation to the amounts of neuropeptide-like immunoreactivity detected. Extracted supernatants of rat masseter muscle were concentrated, lyophilized, and redissolved in 200 µL distilled water. In addition, reverse-phase HPLC was performed using Genesis C18 column (Jones Chromatography) eluted for 60 minutes with a linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. Samples were passed through Millipore GS filters (0.45 µm; Sep-Pak) before being injected onto the column. Synthetic rat CGRP (Neosystem) was used as a standard. Gilson Unipoint software controlled 2 Gilson 306 HPLC pumps. A gradient of 10% to 60% acetonitrile was used. Samples of 1 mL were collected at an elution rate of 1 mL/min.

Each sample was lyophilized and redissolved in 100-µL sodium phosphate buffer (pH 7.4) before analysis. The samples were assayed for immunoreactivity in the tubes used for their collection. The detection limit for the concentrated sample was 7.8 pmol/L.

Statistical analysis was carried out using the Mann-Whitney test (SPSS software 10.0). $P < .05$, $P < .01$ and $P < .001$ were considered to reflect statistical significance.

Results

After 9 to 12 days, observable signs of inflammation (redness, swelling, and increased cutaneous temperature) were seen in the skin overlying the injected right masseter muscle in the rats challenged with Freund's adjuvant. No signs of inflammation were seen in the skin overlying the masseter muscles of the saline-treated rats. Compared to the control rats, scratching behavior was significantly different in the myositis group at 12 days (15 ± 4; $P < .01$), and myositis-group rats consistently scratched more than control-group rats throughout the observation period (Fig 1). The difference in scratching between the groups reached peak levels at day 15 (20 ± 3) and decreased toward the end of the experiment at day 21 (7 ± 2). In contrast, there was no increase in scratching behavior in the control group during the observation period.

The amounts of CGRP-LI (Fig 2) in the right masseter muscles of the myositis group were greater after 21 days (97 ± 39 pmol/g) than the amounts in the saline-treated rats (right side, 62 ± 14 pmol/g; left side, 60 ± 14 pmol/g). The highest concentration of CGRP-LI (135 ± 66 pmol/g) was found in the contralateral masseter muscle in the myositis group. Reverse-phase HPLC analysis of the masseter muscles (Fig 3) showed that the CGRP-LI was eluted in the position of the corresponding synthetic rat CGRP. The CGRP-LI was 62% lower ($P < .05$) in the hypothalamus of the myositis group (280 ± 40 pmol/g) compared to the controls (737 ± 208 pmol/g). However, in the pituitary gland of the myositis group the concentration of CGRP-LI was 32,587 ± 54,177 pmol/g compared to 3,016 ± 654 pmol/g in the control group ($P < .05$) and was 75 ± 62 pmol/g in the blood plasma of the myositis group compared to 40 ± 27 pmol/g in the control group (Table 1). No significant changes were seen in the hippocampus (myositis versus control).

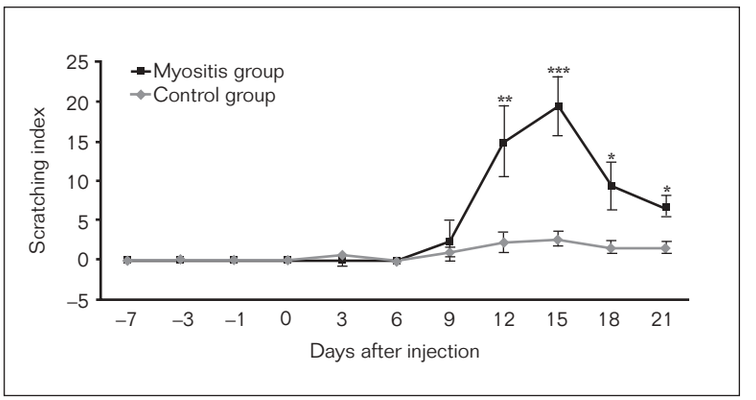


Fig 1 Mean seconds of scratching behavior per 30 minutes (termed scratching index) at consecutive intervals for 21 days. Significant differences between the myositis and control (saline-treated) groups are denoted with * ($P < .05$), ** ($P < .01$), and *** ($P < .001$).

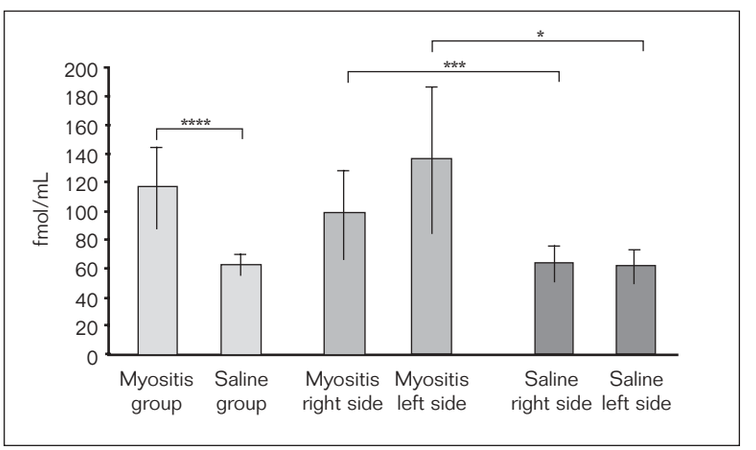


Fig 2 CGRP-LI (pmol/g) in the myositis (Freund's adjuvant–treated) versus control (saline-treated) groups. Significant differences between the myositis and control groups are denoted with * ($P < .05$), *** ($P < .005$), or **** ($P < .001$).

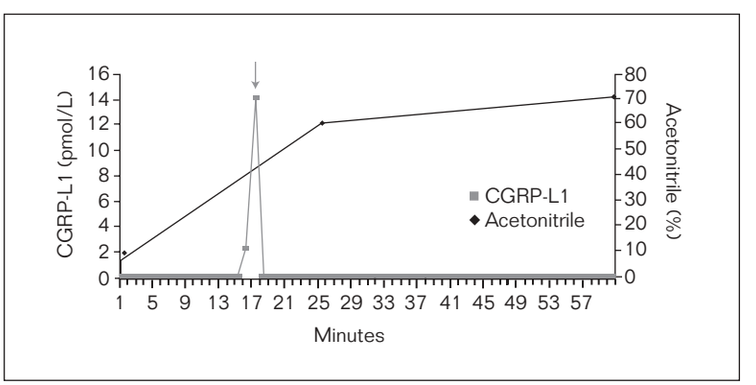


Fig 3 Reverse-phase HPLC of CGRP-LI in extract from masseter muscle in rat. The main peak is eluted in the position of the corresponding synthetic peptide *(arrow)*. Acetonitrile increased over time from 0% to 70%.

Challenge with Freund's adjuvant resulted in edema formation, invasion of inflammatory cells into the muscle tissue, and a high density of macrophages in the connective tissue compared with the saline-treated rats as shown by H&E staining (Figs 4a and 4b). CGRP-immunoreactive nerve fibers were detected mainly in the blood vessel walls and in close proximity to the muscle cells. A higher number of CGRP-IR fibers were detected in the inflamed rat masseter muscles than in the muscles of saline-treated rats (Figs 5a to 5c).

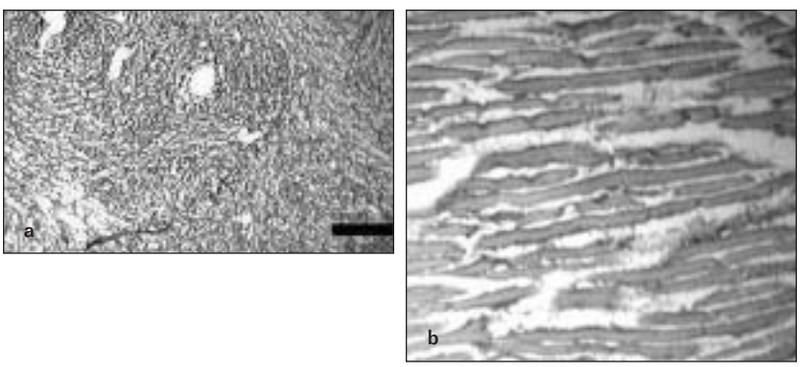
Discussion

In this study CGRP-LI increased bilaterally in the masseter muscles after induction of unilateral masseter myositis. Increased CGRP-LI in the neuropituitary and decreased CGRP-LI in the hypothalamus compared to the saline-treated rats was also demonstrated. A broad variety of biological functions have been ascribed to CGRP-LI, such as nociception,[15] microvascular reactions,[1] and increased glucose uptake and glycolysis in the skeletal muscles.[16] Previous studies have shown

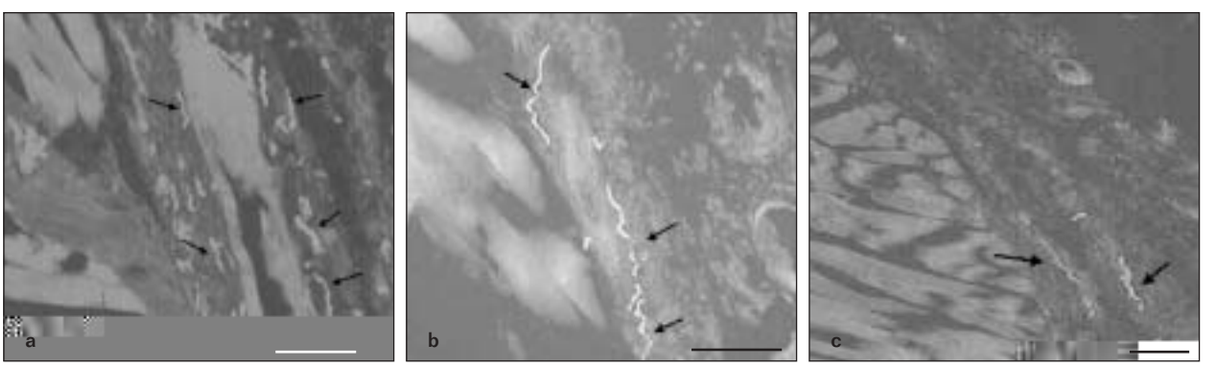
Table 1 Calcitonin Gene-Related Peptide (pmol/g) in the Hippocampus, HPS-Axis, and Blood Plasma

Tissue sample	Saline-treated	FA-treated	*P*
Hippocampus	262 ± 109	243 ± 57	NS
Hypothalamus	737 ± 208	280 ± 40	*
Adreno-pituitary	9574 ± 2722	9904 ± 5425	NS
Neuro-pituitary	3016 ± 654	32,587 ± 54,177	*
Medial eminence	1999 ± 176	2377 ± 172	NS
Blood plasma	40 ± 27	75 ± 62	*

*Significant differences between the myositis group, ie, rats treated with Freund's adjuvant (FA), and saline-treated controls ($P < .05$).
NS = nonsignificant differences.



Figs 4a and 4b Micrographs of H&E-stained sections through the masseter muscle. There was a larger number of inflamed cells in the Freund's adjuvant–treated muscle *(a)* than in the saline-treated muscle *(b)*. Bar = 100 μm.



Figs 5a to 5c Immunofluorescence micrographs of 3 longitudinal sections through the masseter muscle after incubation with antiserum to CGRP. Arrows denote nerve fibers. CGRP-LI was found in the wall of the vessels *(a)* and in the connective tissue close to the muscle *(b)*. More CGRP-LI was found in Freund's adjuvant–injected muscle than in saline-injected muscle *(c)*. CGRP-LI positive nerve fibers *(arrows)* were arranged in nerve bundles close to the muscle and also occurred around a blood vessel wall. Bar = 100 μm.

that local inflammation elevates the CGRP-LI levels in the peripheral tissues.[1] Increased concentrations of CGRP-LI in rats with experimentally induced myositis suggest that CGRP-LI is involved in the inflammatory process.[1,6] The inflammatory response may not be due to the increase of CGRP-LI in the masseter muscles alone, since the contralateral side displayed no signs of inflammation concomitant with a local increase of CGRP-LI. A higher number of round cells were seen in the Freund's adjuvant–injected myositis muscle compared to the contralateral side. Moreover, CGRP is synthesized and secreted by T-lymphocytes that it then regulates.[17] Lymphocyte-derived CGRP may act in an autocrine/paracrine mode and play an important role in inflammatory conditions. Since CGRP has been considered both an inflammatory[1] and anti-inflammatory mediator,[18] it is possible that in this study CGRP may have acted in a dose-dependent manner.[19]

Conspicuously higher levels of CGRP-LI were found in the contralateral muscles in the myositis

group in comparison to the ipsilateral side and the saline controls. The reason for this increase is not fully understood although bilateral effects following unilateral induction of inflammation have also been demonstrated in recent studies.[20] Yu et al demonstrated that intrathecal administration of the CGRP antagonist CGRP8-37 reduces the withdrawal latency bilaterally after unilaterally induced hindpaw inflammation.[3] Such bilateral responses to a unilateral inflammatory process indicate raised activity in the spinal cord[6] and the probable involvement of central mechanisms.[21,22] Amman et al[23] have also reported that unilateral intraplantar injection of nerve growth factor resulted in bilateral increases in the expression and synthesis of CGRP.

Previous studies have revealed elevated CGRP levels in different brain regions after peripheral ischemia had been induced.[24] The current study found raised levels of CGRP-LI in the pituitary gland concomitant with the increase in the masseter muscles. In contrast, CGRP-LI was decreased in the hypothalamus of the myositis group. A possible explanation for these changes could be that rats with chronic myositis develop an abnormal function of the HPA axis[25,26] triggered by inflammation.[24]

Peripheral inflammation induces avoidance behavior, such as back-paw scratching in the rat, which is seen as a sign of presumed nociception.[27–29] In this study, this assumed nociceptive behavior in Freund's adjuvant–treated rats was significantly different from the control group at day 12 and lasted through the end of the experimental period, possibly reflecting disturbed activity in the HPA axis. Indeed, this scratching behavior can be influenced by several mechanisms, including endogenous opioids. For example, in a recent study,[30] a subcutaneously administered opioid analog reduced rat facial rubbing-scratching behavior in a dose-dependent manner. Also, unilateral hindpaw inflammation induced by Freund's adjuvant is associated with a significant upregulation of opioid binding sites, mostly in small-diameter nociceptive dorsal root ganglion neurons.[31]

In conclusion, central neural changes as well as bilateral masseter changes in CGRP-LI levels were observed 3 weeks after the induction of unilateral masseter inflammation, suggesting that CGRP may be one of the mediators of experimentally induced myositis in the masseter muscle of the rat.

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