Effects of Adjuvant on Neuropeptide-Like Immunoreactivity in the Temporomandibular Joint and Trigeminal Ganglia

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To study the role of the nervous system in temporomandibular joint arthritis, substance P-, calcitonin gene-related peptide-, and neuropeptide Y-like immunoreactivity in the trigeminal ganglia and temporomandibular joint of rats was examined. Arthritis was induced in female Lewis rats through bilateral injection of a suspension of heat-killed Mycobacterium butyricum *in paraffin oil into the temporomandibular joint. Control rats received paraffin oil via the same route. Tissues were collected for neuropeptide extraction 28 days after injection and analyzed by radioimmunoassay and reverse-phase high-performance liquid chromatography. Calcitonin gene-related peptide was significantly increased in the arthritic trigeminal ganglia. Substance P, calcitonin gene-related peptide, and neuropeptide Y in the arthritic temporomandibular joint were significantly increased as compared to controls. The results of this study show that sensory and sympathetic neuropeptides may possibly be associated with the development of arthritis in the temporomandibular joint of rats.*
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key words: adjuvant arthritis, calcitonin gene-related peptide, immunoreactivity, neuropeptide Y, radioimmunoassay, substance P, temporomandibular joint, trigeminal ganglia

The significance of assessing neuropeptides in joint tissue relates to numerous observations suggesting an involvement of the nervous system in the pathophysiology of inflammatory joint disease. In fact, both the sensory[1,2] and sympathetic[3] nervous systems have been implicated. Many sensory neurons synthesize both substance P (SP) and calcitonin gene-related peptide (CGRP).[4,5] In sympathetic neurons, neuropeptide Y (NPY) is synthesized and coreleased with norepinephrine.[6] It has been previously suggested that NPY is involved in the regulation of inflammatory processes in the human temporomandibular joint (TMJ) as it correlates with TMJ pain and dysfunction.[7,8] It has also been shown that the increased NPY concentrations in the TMJ of patients suffering from rheumatoid arthritis were associated with reduced intraarticular temperature, suggesting that NPY mediates the reduction of the microcirculation in the arthritic TMJ.[9] Increased concentrations of SP have also been detected in the arthritic TMJ, which would mean that sympathetic fibers, as well as sensory fibers, are activated.[8]

The present authors have recently reported that acute experimentally induced TMJ arthritis in rats[10–14] results in pronounced changes in neuropeptide-like immunoreactivity (-LI) in the synovial fluid. Considering that neuropeptide concentrations in the blood are very low, the contribution of neuropeptides from the blood circulation of the TMJ synovial fluid is negligible.[9] Therefore, the major portion of the neuropeptides in the synovial fluid is most likely of neuronal origin.[15,16] However, the possibility of a nonneuronal origin cannot be excluded.[17] The aim of the present study was to elucidate the contribution of the nervous system by measuring the concentration of CGRP-LI, SP-LI, and NPY-LI in the TG and TMJ affected by adjuvant-induced arthritis.

Materials and Methods

The study was performed on 24 female Lewis rats weighing between 230 and 250 g. The animals were housed five per cage at 21°C in a 12-hour light-dark cycle and were given water and pellets ad libitum. Arthritis was induced in 12 of the rats through bilateral intraarticular injection (0.01 mL) of a suspension of heat-killed mycobacteria in paraffin oil (10 mg/mL) (adjuvant arthritis [AA]) into the TMJ. The 12 intact control rats received 0.01 mL of paraffin oil in the same manner. The rats were killed by decapitation under ether anesthesia 28 days after inoculation. The TMJ, including the capsule and the synovial membrane, was dissected bilaterally separately, and the TG was excised, yielding two samples per rat. Each frozen tissue sample was weighed before extraction. The neuropeptides were extracted and quantified in the manner recently described for bone and joint tissue.[15] Prior to extraction, the tissues were cut into small pieces, boiled for 10 minutes in 2 mol/L acetic acid in 4% ethylenediaminetetraacetic acid (EDTA), homogenized in a Polytron (15 seconds), sonicated (30 seconds), and centrifuged at 3000 rpm × 9 G for 15 minutes. The supernatants were lyophilized and diluted in a 2-mL RIA buffer. These samples were further diluted 1:10 and kept at –20°C until analysis.

Analysis

Calcitonin gene-related peptide was analyzed using antiserum CGRP8 raised in a rabbit against conjugated rat CGRP. High-performance liquid chromatography– (HPLC) purified ^{125}I-Histidyl rat CGRP was used as radioligand and rat CGRP as standard. The detection limit of the assay for rat CGRP was 1 fmol/mL, and crossreactivity of the assay to SP, neurokinin A, neurokinin B, neuropeptide K, gastrin, neurotensin, bombesin, neuropeptide Y, and calcitonin was less than 0.01%. Crossreactivity with human CGRP α and β was 93% and 24%, respectively, and with rat CGRP α and β 100% and 120%, respectively. Intra- and interassay coefficients of variation were 8% and 14%, respectively.

Substance P was assessed using antiserum SP2[15] raised in a rabbit against bovine serum albumin-(BSA) conjugated rat SP. The antiserum reacts with SP and SP sulfoxide, but not with other tachykinins in the concentration range 7.8 fmol/mL to 3200 fmol/mL. High-performance liquid chromatography–purified rat ^{125}I-Tyrosine SP was used as radioligand, and rat SP was used as standard.[15] The detection limit was 1 fmol/mL. Intra- and interassay coefficients of variation were 7% and 11%, respectively.

Neuropeptide Y was analyzed using antiserum Nl, which crossreacts 0.1% with avian pancreatic polypeptide but not with other peptides.[15] The detection limit of all the assays was 11 fmol/mL. Intra- and interassay coefficients of variation were 7% and 12%, respectively.

Reverse-phase HPLC was applied to extracts of normal as well as arthritic TMJs to characterize the neuropeptides studied. Extraction in 2 mol/L acetic acid in 4% EDTA was found to provide optimum yield of both sensory and autonomic neuropeptides. A Waters Delta Pak C18 300 A 3.9-mm × 150-mm column (Wallac Sverige AB, Upplands Väsby, Sweden) was used, and elution was performed with a 40-minute linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. Two Pharmacia P3500 HPLC pumps were controlled by a Pharmacia GP250 gradient programmer (Pharmacia Biotech Norden, Sollentua, Sweden). A gradient of 20% to 40% acetonitrile was used for SP, whereas a gradient of 20% to 50% was used for CGRP and NPY. These samples were passed through Millipore GS filters (0.45 μm) (Millipore AB, Sundbyberg, Sweden) before chromatography to remove particulate matter, and 200 μL of each sample was injected into the column. Fractions of 0.5 mL were collected at an elution rate of 1.0 mL/minute. Each fraction was lyophilized and reconstituted in 100 μL in distilled water before analysis. The fractions were assayed for immunoreactivity in the same tubes in which they were collected. High-performance liquid chromatography analysis of the immunoreactive material from joint samples with regard to SP,

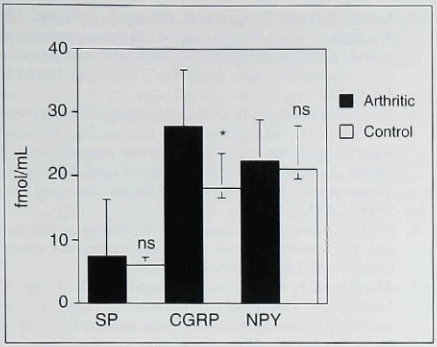

Fig 1 Substance P- (SP), calcitonin gene-related peptide- (CGRP), and neuropeptide Y- (NPY) like immunoreactivity in the arthritic and in the control trigeminal ganglia (N = 8; mean ± SEM. $*P < .05$, $**P < .01$, $***P < .005$; ns = nonsignificant).

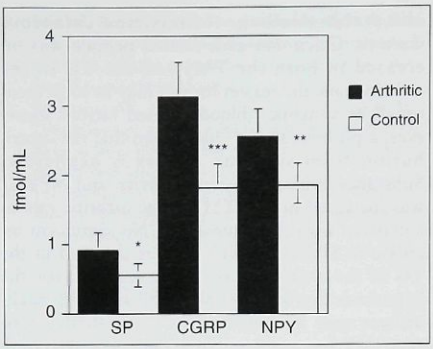

Fig 2 Substance P- (SP), calcitonin gene-related peptide- (CGRP), and neuropeptide Y- (NPY) like immunoreactivity in the arthritic and in the control temporomandibular joint (N = 8; mean ± SEM. $*P < .05$, $**P < .01$, $***P < .005$).

CGRP, and NPY consistently resulted in a main peak eluted in the position of the corresponding synthetic peptide. Thus, no evidence of multiple immunoreactivities was noted for SP, CGRP, or NPY.

Data were tested for normalcy, and evidence was found that they were non-Gaussian in most instances. Therefore, the nonparametric Wilcoxon's matched ranked test and the Mann-Whitney *U* test were used to analyze differences between the two groups, and Spearman's rank correlation coefficient was used to analyze correlation between variables. *P* values < .05 were considered significant.

Results

Effects of Adjuvant Arthritis on Neuropeptide-Like Immunoreactivity in the Trigeminal Ganglia

In the TG of the arthritic rats, CGRP-LI was increased by 54% as compared to controls. Substance P-like immunoreactivity and NPY-LI showed no significant differences as compared to controls (Fig 1).

Effects of Adjuvant Arthritis on Neuropeptide-Like Immunoreactivity in the Temporomandibular Joint

In the TMJ of the arthritic rats, significant increases of SP-LI, CGRP-LI, and NPY-LI concentrations, as compared to controls, were detected. Substance P-like immunoreactivity was increased by 62%, CGRP-LI by 72%, and NPY-LI by 38% (Fig 2).

Correlation Between Neuropeptide-Like Immunoreactivity in the Temporomandibular Joint and in the Trigeminal Ganglia

In the arthritic group, SP correlated by $r = -.15$ ($P = .72$), CGRP by $r = -.01$ ($P = .98$), and NPY by $r = .03$ ($P = .95$) between the TMJ and the TG. In the control group, SP correlated by $r = .96$ ($P = .002$), CGRP by $r = .2$ ($P = .63$). and NPY by $r = .097$ ($P = .82$) between TMJ and TG.

Discussion

In the present study, CGRP-LI was increased in both the arthritic TMJ and TG. Substance P-like immunoreactivity and NPY-LI increased only in the TG, but there was a tendency towards an increase in the TMJ. Adjuvant arthritis, brought about by the injection of mycobactericum into the rat, is used as a chronic animal model in the study of human rheumatoid arthritis.[16,18] It has been demonstrated that adjuvant-induced arthritis is related to the spread of adjuvant to regional lymph nodes.[19] No local signs of inflammation were seen in the joints of the control group injected only with paraffin oil. However, the animals that were given an intraarticular injection of AA developed

observable swelling, redness, and cutaneous warmth. Calcitonin gene-related peptide was increased in both the TMJ and the TG in the arthritic rats; the reason for this may be local (neuronal) or systemic- (blood) related factors. However, a previous study[20] has shown that the contribution from systemic factors is negligible. Substance P-like immunoreactivity and NPY-LI was increased in the TMJ of the arthritic rats as compared with the control rats. No significant increase in SP-LI and NPY-LI was detected in the TG of the arthritic rats. These results favor the hypothesis that local peripheral SP and NPY mechanisms may be involved in the arthritic process.[2,15,21] It is not possible to determine the origin of the SP-LI and NPY-LI found in the TMJ based on the experimental data of the present study. Perhaps some of the SP-LI and NPY-LI are of neuronal origin, since both peptides have been found in peripheral nerves.[6,22] However, local factors may also contribute. The local increase of NPY may be the result of an increased synthesis of Nerve Growth Factor (NGF) at the site of inflammation.[17,23] This suggestion is supported by a study showing that NGF may induce a production and release of NPY by T-lymphocytes.[17] Injection-induced adjuvant arthritis triggers an increase of SP-LI, CGRP-LI, and NPY-LI in TG and TMJ.

The results of the present study show that the alteration in neuropeptide-like concentrations in TG and TMJ may reflect neuronal-induced arthritis or arthritis-induced neural activation.

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Resumen

Los Efectos de un Facilitador sobre la Inmunoreactividad de un Péptido Parecido al Neuropéptido en la Articulación Temporomandibular y los Ganglios Trigéminos

Se estudió la interacción del sistema nervioso examinando la Substancia P, el Péptido Relacionado al Gen de la Calcitonina, y la inmunoreactividad de un péptido parecido al Neuropéptido Y, en los ganglios trigéminos y las articulaciones temporomandibulares (ATMs) de ratas con artritis experimental. La artritis fue inducida en ratas hembras tipo Lewis por medio de una inyección bilateral (en las ATMs de las ratas) de una suspensión de Mycobacterium butyricum muerto a base de calor en aceite de parafina. Las ratas de control recibieron aceite de parafina a través de la misma ruta. Se recolectaron los tejidos para la extracción del neuropéptido y analizados por medio de radioinmunoensayos y de cromatografía líquida de alto rendimiento y de fase inversa. El Péptido Relacionado al Gen de la Calcitonina aumentó en los ganglios trigéminos artríticos, y la Substancia P, el Péptido del Gen relacionado a la Calcitonina, y el Neuropéptido Y aumentaron en la ATM artrítica en comparación con los controles. Los resultados de este estudio demuestran que hay una interacción cercana entre el sistema nervioso y el desarrollo de la artritis en la ATM de la rata.

Zusammenfassung

Helfende Auswirkungen auf neuropeptidähnlicher Immunreaktivität im Kiefergelenk und in den Trigeminusganglien

Die Interaktion des Nervensystems wurde anhand der Untersuchung der Substanz P, Calcitonin Gene-Related Peptid und Neuropeptid Y-ähnlicher Immunreaktivität in den Trigeminusganglien und Kiefergelenken (TMJs) von Ratten während experimenteller Arthritis studiert. Eine Arthritis wurde in weibliche Lewisratten durch eine bilaterale Injektion einer Suspension hitzeabgetöteter Myobacterium butyricum in Paraffinöl in die Kiefergelenke der Ratten ausgelöst. Kontrollratten erhielten Paraffinöl durch denselben Weg. Gewebe wurden gesammelt für die Extaktion von Neuropeptiden und wurden analysiert mittels Radioimmunoassay und Umkehrphasen-Hochleistungsflüssigkeitschromatographie. Calcitonin Gene-Related Peptid nahm in den arthritischen Trigeminusganglien zu, und Substanz P, Calcitonin Gene-Related Peptid, sowie Neuropeptid Y nahmen zu in den arthrithischen Kiefergelenken verglichen mit den Kontrollen. Die Resultate dieser Studie zeigen, dass es eine nahe Interaktion zwischen dem Nervensystem und der Entstehung einer Arthritis in den Kiefergelenken der Ratte gibt.