A MODEL FOR EXPERIMENTAL INDUCTION OF ACUTE TEMPOROMANDIBULAR JOINT INFLAMMATION IN RATS: EFFECTS OF SUBSTANCE P(SP) ON NEUROPEPTIDE-LIKE IMMUNOREACTIVITY

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Summary

This is a study of neurokinin A (NKA)-, calcitonin gene-related peptide (CGRP)- and neuropeptide Y (NPY)- like immunoreactivity (LI) in the cerebrospinal fluid (CSF), plasma and perfusates (PF) from the temporomandibular joints (TMJs) of the rat during acute inflammation. Substance P (10^{-5} M, 0.01 ml) was injected into the right TMJ of the rat. The TMJs of the control rats, were injected with 0.01 ml saline. CSF, plasma and PF from TMJs were taken at 2, 6 and 24 hrs following injection. The neuropeptide-LI level was analysed by specific radioimmunoassays and compared with control values. Unilateral injection of SP into the rat TMJ resulted in a general increase in the concentration of NKA-, CGRP- and NPY-LI from the temporomandibular joints (TMJs) of the rat during acute inflammation. In the CSF NKA- and CGRP-LI was increased leaving the NPY-LI unaffected. In general no changes in peptide concentrations were seen in plasma. The results indicate that SP directly or indirectly induces a local release of peptides through an action at sensory and sympathetic neurons.

Key Words: inflammation, substance P, neurokinin A, calcitonin gene-related peptide, neuropeptide Y

In the search of neuronal messenger presumed to be involved in inflammation and arthritis, significant interest has been focused on neuropeptides (12,15). A wide variety of neuropeptides have been identified in the sensory and autonomic nervous system. In the sensory nervous system substance P (SP) (20) and neurokinin A (NKA) (14) have been found in afferent nerves and shown to be related to the transmission of presumed nociceptive information. Calcitonin gene-related
peptide (CGRP) (7), co-exist with and potentiates the effects of SP and NKA in nociception. Furthermore, SP (24,29), NKA (8) and CGRP (8,34) have been implicated in vasodilatation and immune regulation. Experimentally, SP infusion into the joint cavity has been shown to induce arthritis (21), which was reversed by capsaicin treatment (15). In the autonomic nervous system neuropeptide Y (NPY) have been shown to exert a vasoactive as well as an immunoregulatory role (22). Also, the concentration of NPY was raised in the synovial fluid of patients with rheumatoid arthritis (1-6,18,19), and in experimentally induced arthritis (22), supporting a role for NPY in inflammation. There is evidence that classical transmitters such as acetylcholine, catecholamines and neuropeptides are released differently depending on the mode of stimulation (25). Also, co-localization of neuropeptides, and also different neuropeptides has been demonstrated (14) implicating a variety of functional interactions.

We have previously reported on increased levels of SP, NKA, CGRP and NPY in the synovial fluid of patients suffering from TMJ arthritis (1-4) indicating that the nervous system is playing a role in the pathophysiological process. However, knowledge about the contribution of the nervous system in TMJ monoarthritis is limited.

The aim of the present study was to elucidate the local and general interaction between SP and the sensory and sympathetic nervous system by measurement of NKA, CGRP and NPY in temporomandibular joint perfusates, cerebrospinal fluid and plasma.

**Methods**

The study was carried out on 80 male albino Sprague-Dawley rats, weighing 250-300 g. The rats were allowed to become habituated to the laboratory for at least 7 days before experimentation. All rats were maintained under identical conditions, which included alternate cycles of 12 hrs light and 12 hrs darkness, environmental temperature of 24°C, 60% relative humidity, food and water ad libitum.

On the day of the experiment the rats were anaesthetized with chloralhydrate (0.4 g/kg) intraperitoneally. The skin overlying the TMJs was shaved before an intraarticular injection. The choice of dose of SP was based on preliminary trials (unpublished observations) showing that 0.01 ml of SP $10^{-5}$ M into one TMJ induced monoarthritis as defined by histological analysis (hematoxylin-eosin). In the TMJs that had been subjected to SP injection there were synovial hyperthrophy, oedema and round cell infiltrate. No signs of inflammation were seen in any other joint (hip, knee or ankle). 40 rats were given substance P (SP) 0.01 ml $10^{-5}$ M via a 27-gauge needle into the right TMJ. Another group of 40 control rats were injected with 0.01 ml saline. After a period of 2 (28 rats; 14 injected with SP and 14 with saline), 6 (24 rats; 12 injected with SP and 12 with saline) or 24 (28 rats; 14 injected with SP and 14 with saline) hours following the injection, the rats were again anaesthetized with chloralhydrate. A 2 cm longitudinal skin incision was made exposing the TMJ. Another group of 40 control rats were injected with 0.01 ml saline. After a period of 2 (28 rats; 14 injected with SP and 14 with saline), 6 (24 rats; 12 injected with SP and 12 with saline) or 24 (28 rats; 14 injected with SP and 14 with saline) hours following the injection, the rats were again anaesthetized with chloralhydrate. A 2 cm longitudinal skin incision was made exposing the TMJs. A 27-gauge needle was inserted into each joint. The right joint was perfused with 0.01 ml 0.9% saline through the 27-gauge needle using a push and pull technique. Collection was carried out for 15 minutes. For collection of CSF, following TMJ perfusion, the rats were placed in a stereotaxic frame. The atlanto-occipital membrane was exposed by retracting the overlaying muscles and samples of 80-150 ml of CSF were obtained through a 27 gauge needle connected with a 1 ml syringe via a polyethylene tubing. Blood (1.5-4.5 ml) was then collected by a puncture of the heart with a vacutainer tube containing heparin 143 IU and Trasylol 500 IU ml$^{-1}$. The samples were centrifuged and the plasma was removed and frozen. All samples were rapidly cooled and stored at -80°C until analysis. Samples from the CSF, plasma and PF
were extracted using a reverse-phase C18 cartridge (Sep Pak, Waters) and analysed using competitive radioimmunoassays (33). The analysis was performed using the following antisera: Neurokinin A-like immunoreactivity (NKA-LI) was done using antiserum K12 which reacts with NKA (100%), NKA (3-10) (48%), NKA (4-10) (45%), neurokinin B (26%), neuropeptide K (61%) and eledoisin (30%), but not with SP (31,32,33).

Calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) was analyzed using antiserum CGRP8 raised in a rabbit against conjugated rat CGRP (31, 33). HPLC purified [125I]-histidyl rat CGRP was used as radioligand and rat CGRP as standard. The detection limit of the assay for rat CGRP was 9 pmol/l and cross-reactivity of the assay to SP, neurokinin A, neurokinin B, neuropeptide K, gastrin, neurotensin, bombesin, neuropeptide Y and calcitonin was less than 0.01%. Cross-reactivity 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.

Neuropeptide Y-like immunoreactivity (NPY-LI) was analyzed using antiserum N1, which cross-reacts 0.1% with avian pancreatic polypeptide but not with other peptides (32). The detection limit of the assay was 11 pmol/l. Intra- and interassay co-efficients of variation were 7% and 12%. The lower detection limit in all extracted samples was 0.1 fmol/ml for all assessed peptides. Reverse-phase HPLC was applied to the samples to characterize the neuropeptides of interest. 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 x 150 mm column was used and elution was performed with a 40 min linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. Two Pharmacia P3500 HPLC pumps were controlled by a Pharmacia GP250 gradient programmer. A gradient of 20-40% acetonitrile was used for NKA, whereas a gradient of 20-50% was used for CGRP and NPY. These samples were passed through Millipore GS filters (0.45 μm) 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/min. Each fraction was lyophilized and reconstituted in 100 μl. I distilled water before analysis. The fractions were assayed for immunoreactivity in the same tubes used for their collection.

Data were tested for normality and evidence was found that the data were Gaussian in most instances. Therefore, the non-parametric Wilcoxon’s matched ranked test and Mann-Whitney U-test were used to analyse differences between two groups and Spearman’s rank correlation coefficient to analyze correlation between variables. P values < 0.05 were considered significant.

Results

TMJ Perfusates

In the TMJ perfusates of the SP injected group there was a significant increase of NKA-LI as compared to the saline treated group at 2 and 6 hrs. CGRP-LI was increased at 2, 6 and 24 hrs and NPY-LI at 6 and 24 hrs, Fig.1.

Cerebrospinal Fluid

In the CSF of the SP injected groups there was a significant increase of NKA- and CGRP-LI at 2, 6 and 24 hrs. On the other hand there was no change in NPY-LI in the CSF 2, 6 and 24 hrs as compared to the saline injected group, Fig.2.
Changes of NKA-, CGRP- and NPY-LI in temporomandibular perfusates (TMJ PF) after injection of substance P (SP) into the TMJ compared to a control group. ■ denotes SP and □ denotes Saline.

* p<0.05, ** p<0.01 and *** p<0.001.
Changes of NKA-, CGRP- and NPY-LI in cerebrospinal fluid (CSF) after injection of substance P (SP) into the TMJ compared to a control group. ■ denotes SP and □ denotes Saline. *p<0.05, **p<0.01 and ***p<0.001.
Plasma

In plasma NKA-LI was significantly reduced in comparison with controls after 24 hrs and NPY-LI was significantly increased in the SP group at 2 hrs compared to controls. No significant changes in CGRP-LI was seen in plasma following SP injection, Fig. 3. Furthermore, no changes in SP-LI were seen at 2, 6 or 24 hrs (data not shown).

HPLC

HPLC analysis of the immunoreactive material from joint samples with regard to CGRP, NPY and NKA consistently resulted in a main peak eluting in the position of the corresponding synthetic peptide (data not shown). Thus, no evidence of multiple immunoreactivities was noted for CGRP, NPY and NKA.

Discussion

The results of the present study show that an intra-articular injection of SP into one TMJ cause a significant increase in NKA-, CGRP- and NPY-LI in the TMJ PF as compared to saline. These results are in line with the increased concentrations of NKA-LI, CGRP-LI and NPY-LI occuring in the synovial fluid of patients suffering with arthritic TMJs (16,19).

Intra-articular injection of SP resulted in enhanced NKA- and CGRP-LI in CSF as early as 2 hrs after injection and still being present at 24 hrs. NPY-LI concentrations in the CSF was not affected by the SP injection. The increased concentrations of NKA-LI and CGRP-LI but not NPY-LI in CSF, and all three peptides in TMJ PF, indicates that there are different release patterns in sensory and sympathetic neurons. This is supported by a study showing that SP has a direct excitatory effect on sensory but not sympathetic neurons (17). NKA and CGRP have been found to be co-localized in sensory neurons (27,30) and the our results indicate that they are simultaneously released, peripherally as well as centrally, following stimulation with SP.

In plasma only two significant changes were seen; one was the significant decrease of NKA-LI 24 hrs after SP-injection and the other was the significant increase in NPY-LI 2 hrs after the injection, the latter possibly being part of a stress response. It may be suggested that the decrease of NKA-LI in plasma after 24 hrs is due to an increased synthesis of endopeptidase triggered by the SP injection (11). Data on the role of autonomic transmitters in inflammation is limited. It has been demonstrated that sympathectomy mitigates the inflammatory signs (21,22). It has also been shown that the severity of adjuvant arthritis is reflected by an increase in circulating adrenaline (9), which is supposed to elicit the release of proinflammatory mediators from postganglionic sympathetic fibres. Furthermore, it has been demonstrated that NPY interact with activity in sensory nerves modulating the release of SP (27).

Taken together the results of the present study show that an unilateral injection of SP into the rat TMJ results in a general increase in the concentration of NKA-, CGRP- and NPY-LI in the TMJ PF at 2, 6 and 24 hrs following injection. In the CSF NKA- and CGRP-LI was increased leaving the NPY-LI unaffected. In general no changes in peptide concentrations were seen in plasma.

The results indicate that SP directly or indirectly induces a local release of peptides through an action at sensory and sympathetic neurons (10, 23). The increased concentrations of NKA- and
Changes of NKA-, CGRP- and NPY-LI in plasma after injection of substance P (SP) into the TMJ compared to a control group. ■ denotes SP and □ denotes Saline. * p<0.05, ** p<0.01 and *** p<0.001.
CGRP-LI in CSF likely reflects an increased afferent activity in the sensory neurons innervating the joint. The finding that SP was not increased in plasma at 2, 6 and 24 hrs support the hypothesis that the results obtained were due to local changes and not due to SP escaping from the joint.

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**References**