NEUROPEPTIDES IN THE SALIVA OF HEALTHY SUBJECTS

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Summary

Five neuropeptides: Substance P (SP), Neurokinin A (NKA), Calcitonin Gene-Related Peptide (CGRP), Neuropeptide Y (NPY) and Vasoactive Intestinal Polypeptide (VIP), were measured in the saliva of eight subjects. The saliva was collected using different stimulation techniques: whole resting saliva, whole paraffin stimulated saliva, whole citric acid stimulated saliva and parotid saliva of different secretion rates - 0.25 mL/min, 0.50 mL/min and 1.00 mL/min, also stimulated by citric acid. The neuropeptides were analysed by radioimmunoassay. The results showed that the concentration of all neuropeptides decreased significantly, two- to four-fold (CGRP up to 16-fold) in whole saliva, when the salivary secretion rates increased six- to eight-fold due to stimulation. However, the amounts of all neuropeptides released over time into the whole saliva increased two- to five-fold (ten-fold for CGRP) as the volumes of saliva increased due to chewing-stimulation as compared to resting saliva or citric acid stimulated saliva. There was also more CGRP in the resting saliva than in the citric acid stimulated saliva. The concentration of CGRP in the parotid saliva decreased three- to ten-fold when the salivary flow increased, whereas the concentration of NKA increased three- to four-fold and that of NPY almost two-fold under the same conditions. The concentrations of SP and VIP did not change in the different flows of parotid saliva. The release of all neuropeptides in the parotid saliva over time showed significant increases (3-14-fold) when the secretion rates increased except CGRP, which showed no changes at all. We concluded that neuropeptides are continuously released into the saliva. Their amounts increase with stimulation, but they are diluted by the increased volume of saliva, and they are also affected by the mode of stimulation - muscular activity leads to a greater release than citric acid stimulation. As the neuropeptides play an important role in the control of salivary secretory mechanisms, their normal occurrence and release are of fundamental importance for the understanding of the function of the salivary glands.

Key Words: substance P, neurokinin A, CGRP, neuropeptide Y, vasoactive intestinal polypeptide

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The functions of the salivary glands are controlled by the autonomic nervous system and influenced by the sensory nervous system. Parasympathetic impulses, which are more prevalent, give a strong increase of salivary flow with a low protein content. They induce contraction of myoepithelial cells as well as cause vasodilatation as a part of the secretory process. Sympathetic impulses, which occur more intermittently, cause a low, protein-rich, viscous salivary flow (1-3). The studies of animal and human innervation have revealed that there are parasympathetic nerve fibres around acinar cells, ducts and blood vessels in the major salivary glands (2). It has also been shown that beside the classic transmitters Noradrenaline and Acetylcholine there are neuropeptides present in the nerve fibres of the autonomic nervous system, in the auriculo-temporal nerve, facial nerve and cervical dorsal root fibres (4). There are numerous nerve fibres containing Vasoactive intestinal polypeptide (VIP) and Neuropeptide Y (NPY) in close association with the acini, ducts and blood vessels. Some Calcitonin gene-related peptide (CGRP) - and Substance P (SP) - containing nerves were mainly localised around blood vessels and ducts (5, 6). VIP-containing nerve fibres were found in close proximity of mucous endpieces and to a lesser extent palatal glands, intercalated ducts of the parotid gland, and excretory ducts of parotid, labial and palatal glands (7-8). The neuropeptides have considerable effect on the salivation. In animal studies it has been found that administration of SP and Neurokinin A (NKA) increase the salivary secretion, SP to a greater extent than NKA. CGRP causes a delayed (1-2 min.) increase of the salivary secretion, also smaller than that produced by SP. VIP produces relatively small amounts of secretion, most from gland. submandibularis, least from gland. sublingualis, as well as an increased release of proteins. SP, CGRP and VIP increase significantly the blood flow in the salivary glands. NPY causes vasoconstriction and inhibits the release of NA. CGRP and VIP also enhance the salivary secretion caused by SP and Acetylcholine (4, 9-12). VIP and SP are potent vasodilators in the human submandibular gland (13). Furthermore it has been shown that VIP potentiates the salivary volume response to Acetylcholine (14). The finding of neuronal VIP in salivary glands, its release upon nerve stimulation and its known effect on local blood flow support the view that VIP is a neurotransmitter in the salivary glands (15, 16). SP has been found in human parotid saliva and it is probable that it derives from parasympathetic and sympathetic nerve terminals (17). The local blood flow is under the influence of the autonomic nervous system and the dilation of the capillaries is controlled to a large extent by neuropeptides, which act as neurotransmitters beside or in co-operation with the classic transmitters Noradrenaline and Acetylcholine (18, 19). Non-adrenergic, non-cholinergic mechanisms may have influence on salivary glands as well as trophic effect. Repeated infusions of SP and VIP are capable of preventing the expected reduction in gland weight in animals, following denervation or liquid diet (20). Neuropeptides are produced in the body of the cell, packed into the large dense-core vesicles and transported to nerve terminals where depletion occurs when prolonged stimulation empties them (21).

There are but few studies that investigate the occurrence of neuropeptides in the human saliva. In 1990, Takeyama et al (22) measured the contents of SP in the saliva and plasma of 10 healthy subjects. According to them the saliva contained 7.1 ± 4.0 pmol/L of SP, which was three times higher than in plasma (pmol = 10^-12). Parris et al (23) compared the content of SP in the saliva from patients with chronic low back pain and from 8 healthy volunteers. They found that there was more SP in the saliva of controls than in that of pain-patients and more SP in saliva than in plasma. However, the method of saliva collection was not well described in either study. In 1990, Nicoldi and Del Bianco (24), measured the release of SP, CGRP and VIP in the saliva of patients suffering from migraine, cluster head-ache and compared that with the saliva of 18 healthy controls. The levels of SP in whole resting saliva of healthy subjects were 35.2 ± 4 pmol/mL, of CGRP - 22.02 ± 1.7 pmol/mL, and 63.3 ± 15.8 pmol/mL of VIP. The healthy controls showed significantly higher CGRP & SP - levels in the saliva than the patients during basal conditions. CGRP-content was lower in the saliva of healthy subjects than in the saliva of patients during cluster head-ache attacks. The levels of SP during basal conditions of migraine sufferers were 15.6 ± 3.4 pmol/mL and 23.1 ± 3.1 for cluster head-ache sufferers. The levels of CGRP during basal
conditions of migraine sufferers were $14.3 \pm 2.5 \text{ pmol/mL}$ and of the cluster headache sufferers $33.4 \pm 7.7 \text{ pmol/mL}$ in whole resting saliva. They reported on a VIP - increase during cluster headache attacks ($174.8 \pm 19.5 \text{ pmol/mL}$) and a decrease during migraine attacks ($23.4 \pm 8.9 \text{ pmol/mL}$), as well as a SP & CGRP - increase during both migraine and cluster headache attacks. SP levels were $41.1 \pm 10 \text{ pmol/mL}$, and CGRP levels were $53.7 \pm 5.2 \text{ pmol/mL}$ during migraine periods and during cluster headache periods the levels of SP were $34.1 \pm 1.9 \text{ pmol/mL}$ and CGRP levels were $53.7 \pm 5.2 \text{ pmol/mL}$. Finally, in 1992, Pikula and co-workers (17) measured the SP-like immunoreactivity in human parotid saliva of 31 healthy subjects. The methods of stimulation and collection of the saliva are well described but there is no mention of the actual salivary flow rates. Their results showed a fluctuation in the occurrence of SP in the parotid saliva between the morning and the evening samples, and a mean of $9.3 \pm 2.0 \text{ fmol/mL}$ ($1 \text{ pmol/L}$equals $10^{15}$).

The aim of this study was to investigate the release of the neuropeptides SP, VIP, CGRP, NKA and NPY into the saliva of healthy humans during different kinds of stimulation. In earlier studies few neuropeptides were investigated under varying conditions. To our knowledge this is the first study of the release of several neuropeptides in human saliva, where the conditions of saliva secretion as well as co-activation and co-release are taken into consideration.

**Materials and methods**

Subjects and saliva sampling: Eight healthy subjects were enrolled in this study - 4 males and 4 females aged between 23 years and 41 years (mean - 31.5 years). All but one were students or dentists at the School of Dentistry at the Karolinska Institute in Huddinge, well acquainted with the salivary flow test procedures used as standard at the Department of Cariology (25). The experimental design was approved by the ethical committee at Huddinge Hospital, prior to the start of the study. The participants were informed about the study in writing and they were asked to refrain from eating, drinking and smoking for at least one hour prior to each experiment. Their saliva was collected according to the standard test at four different occasions: unstimulated salivary flow, paraffin-chewing stimulated whole saliva, whole saliva stimulated with 1% citric acid and parotid saliva, collected with the aid of Lashley cannula (Carlson-Crittenden cannula), stimulated with 1-3% citric acid to produce three different flows - 0.25 mL/min, 0.50 mL/min and 1.00 mL/min (25, 26). Each person participating in the study was tested at approximately the same time of the day considering the differences in saliva production during the day. The time lapse between the four different tests ranged between 24 hours and a couple of weeks. Also, the order of different tests was randomized. The samplings of whole unstimulated saliva, chewing stimulated saliva and citric acid stimulated saliva were repeated two more times in order to find out if any variations occur between different occasions. The collected saliva was weighed in order to obtain precise measurements (1 g was considered to correspond to 1 mL). The saliva samples were collected in small test tubes, containing 1 mL 1M acetic acid in order to neutralise the enzymes that would otherwise destroy the neuropeptides. The samples were kept in ice during the experiment and were frozen to -70°C immediately after the end of each session, awaiting the radio immunoassay tests (RIA), that were to be carried out at a later date.

Peptide analyses: VIP-like immunoreactivity (VIP-LI), SP-LI, NPY-LI, CGRP-LI and NKA-LI were analysed in the saliva samples collected from the subjects during the experiments, using competitive radioimmunoassay (RIA) (27). Substance P (SP-LI) was analysed using antiserum SP2, which reacts with SP sulfoxide, but not with other tachykinins. Intra- and interassay coefficients of variation were 7 and 11%, respectively (28). Neurokinin A (NKA-LI) was analysed using antiserum K12 which reacts with NKA (100%), NKA (3-10) (48%), NKA (4-10) (45%), neurokinin B (26%), neuropeptide K (61%) and eledosin (30%), but not with SP. Intra- and
interassay coefficients of variation were 7 and 12%, respectively (29). Calcitonin gene-related peptide (CGRP-LI) was analysed using antiserum CGRPR8 raised against conjugated rat CGRP. HPLC-purified $^{125}$I-Histidyl rat CGRP was used as radioligand, and rat CGRP as standard. The 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 toward human CGRP alpha and beta was 93 and 24%, respectively, and toward rat CGRP alpha and beta 100 and 120%, respectively. Intra- and interassay coefficients of variation were 8 and 14%, respectively (30). Neuropeptide Y (NPY-LI) was analysed using antiserum N1 which crossreacts 0.1% with avian pancreatic polypeptide, but not with other peptides. Intra- and interassay coefficients of variation were 9 and 12%, respectively (31). Vasoactive intestinal polypeptide (VIP-LI) was analysed using antiserum VIP2 raised against conjugated natural porcine VIP. The antiserum does not react with gastrin, pancreatic polypeptide, glucagon, NPY or neurotensin. Intra- and interassay coefficient of variation were 9 and 13%, respectively. The lower detection limit in all saliva samples was 0.1 fmol/mL for all peptide assessments (32).

Statistical methods: The Friedman's test was used in order to test the differences in neuropeptide-release between the different saliva stimulation modes and between the three different flows of parotid saliva, as well as to ascertain the comparability of the whole saliva samples that were taken on three different occasions. The concentration of neuropeptides in pmol/L as well as their amounts produced/min were compared. The differences where $p < 0.05$ were considered significant. Bonferroni's correction for multiple comparisons has been used in order to decrease the alpha level. The significant changes are indicated by (sign.) in the results section. Correlation test was carried out using Spearman rank order correlation.

Results

The occurrence of all the investigated neuropeptides in the saliva of healthy subjects in connection with different salivary flow rates is shown in TABLE I, described as concentration (in pmol/L) and as function of time (in pmol/min). The results are also visualised in Fig. 1.

### TABLE I

The Concentrations of all Investigated Neuropeptides in pmol/L and their Amounts in pmol/min in the Saliva of Eight Healthy Subjects during Different Stimulation Procedures (median / range)

<table>
<thead>
<tr>
<th>Salivary secretion rates</th>
<th>SP</th>
<th>NKA</th>
<th>CGRP</th>
<th>NPY</th>
<th>VIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting saliva: pmol/L pmol/min</td>
<td>5.6 / 4.4</td>
<td>6.0 / 20.0</td>
<td>6.2 / 10.5</td>
<td>9.7 / 11.4</td>
<td>12.0 / 15.6</td>
</tr>
<tr>
<td>0.43 / 0.76 mL/min pmol/min</td>
<td>2.5 / 2.4</td>
<td>2.4 / 8.7</td>
<td>2.4 / 3.6</td>
<td>3.9 / 5.6</td>
<td>6.0 / 6.4</td>
</tr>
<tr>
<td>Paraffin-chewing stim. pmol/L pmol/min</td>
<td>3.8 / 6.6</td>
<td>4.0 / 14.2</td>
<td>7.1 / 11.1</td>
<td>8.5 / 11.3</td>
<td>8.1 / 10.4</td>
</tr>
<tr>
<td>2.44 / 3.42 mL/min pmol/min</td>
<td>8.1 / 5.8</td>
<td>8.5 / 36.8</td>
<td>13.6 / 29.9</td>
<td>19.7 / 20.8</td>
<td>17.3 / 15.1</td>
</tr>
<tr>
<td>Citric acid stim. saliva: pmol/L pmol/min</td>
<td>1.6 / 6.5</td>
<td>2.1 / 12.0</td>
<td>0.5 / 1.0</td>
<td>3.3 / 6.2</td>
<td>3.1 / 6.7</td>
</tr>
<tr>
<td>3.03 / 7.55 mL/min pmol/min</td>
<td>5.6 / 10.3</td>
<td>4.4 / 20.0</td>
<td>1.3 / 2.1</td>
<td>10.8 / 10.8</td>
<td>10.0 / 12.2</td>
</tr>
<tr>
<td>Citric acid stim. parotid saliva</td>
<td>0.25 mL/min pmol/L pmol/min</td>
<td>6.0 / 5.6</td>
<td>5.0 / 3.5</td>
<td>3.4 / 10.2</td>
<td>7.9 / 4.3</td>
</tr>
<tr>
<td>0.50 mL/min pmol/L pmol/min</td>
<td>3.4 / 2.5</td>
<td>13.5 / 25.0</td>
<td>1.2 / 2.6</td>
<td>9.1 / 15.2</td>
<td>7.0 / 14.2</td>
</tr>
<tr>
<td>1.00 mL/min pmol/L pmol/min</td>
<td>4.6 / 15.7</td>
<td>16.9 / 24.8</td>
<td>0.4 / 1.2</td>
<td>10.4 / 10.4</td>
<td>9.0 / 9.1</td>
</tr>
<tr>
<td>4.6 / 16.6</td>
<td>16.9 / 16.4</td>
<td>0.4 / 1.2</td>
<td>9.7 / 8.6</td>
<td>7.6 / 9.1</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1

The concentrations of all the investigated neuropeptides in pmol/L and their amounts in pmol/min in the saliva of eight healthy subjects during different stimulation procedures (medians only).

rest = whole resting saliva
chew = paraffin chewing stimulated whole saliva
citric = citric acid stimulated whole saliva

0.25 = parotid saliva of 0.25 mL/min flow
0.50 = parotid saliva of 0.50 mL/min flow
1.00 = parotid saliva of 1.00 mL/min flow

There were no differences in the release of any of the neuropeptides between the three different occasions on which the whole saliva was collected, allowing us to pool these results for the comparisons between the whole resting saliva, the whole chewing stimulated saliva and the whole citric acid stimulated saliva. The statistical analyses of the results showed that there were some interesting features in the release of the neuropeptides at different salivary flow rates. Due to stimulation the salivary flow rates increased 6 times between the resting saliva and the chewing stimulated saliva, 8 times between the resting saliva and the citric acid stimulated saliva and 1.5 times between the chewing stimulated and the citric acid stimulated saliva. The parotid saliva was increased twice and fourfold, respectively, under controlled conditions. The following changes in occurrence of the neuropeptides could be observed:
Substance P (SP)

Whole saliva: The concentration (pmol/L) of SP in the resting saliva was almost twice as high as in the chewing stimulated saliva (sign.) and three-fold higher than in the citric acid stimulated saliva (sign.). The concentration of SP was also twice as high in the chewing stimulated saliva as in the citric acid stimulated saliva (sign.). The amounts of SP increased three-fold in the chewing stimulated saliva (sign.) and two-fold in the citric acid stimulated saliva (sign.) as compared to resting saliva as a function of time. There was 1.5-fold more SP released over time in the chewing stimulated saliva than in the citric acid stimulated saliva (sign.).

Parotid saliva: The concentration of SP did not differ between the three different flows of parotid saliva. There was three-fold more SP released over time in the highest flow than at 0.25 mL/min (sign.), as well as three-fold more than in the 0.50 mL/min flow (sign.). There was no significant difference in the amounts of SP released over time between the lowest flow and the 0.50 mL/min flow.

Neurokinin A (NKA)

Whole saliva: The concentration of NKA in the resting saliva was three-fold higher than in the citric acid stimulated saliva (sign.), and twice as high in the chewing stimulated saliva than in the citric acid stimulated saliva (sign.). The concentration of NKA in the resting saliva was 1.5-fold higher than in the chewing stimulated saliva (sign.). There was three-fold more NKA released over time in the chewing stimulated saliva than in the resting saliva (sign.) and twice as much as in the citric acid stimulated saliva (sign.). There was two-fold more SP released over time in the citric acid stimulated saliva than in the resting saliva (sign.).

Parotid saliva: There was a three-fold higher concentration of NKA in the 0.50 mL/min flow than in the 0.25 mL/min flow (sign.) and the concentration of NKA in the parotid saliva was four-fold higher in the 1.00 mL/min flow than in the 0.25 mL/min flow (sign.). The concentration of NKA was also almost 1.5-fold higher in the 1.00 mL/min flow of parotid saliva than in the 0.50 mL/min flow (sign.). The amounts of NKA released over time were five-fold higher in the 0.50 mL/min flow (sign.) and 14-fold higher in the 1.00 mL/min flow (sign.) than in the 0.25 mL/min flow, whereas there was almost 2.5-fold more NKA released over time in the 1.00 mL/min flow than in the 0.50 mL/min flow (sign.).

Calcitonin gene-related peptide (CGRP)

Whole saliva: The concentration of CGRP was 14-fold higher in the resting saliva than in the citric acid stimulated saliva (sign.) and 16-fold higher in the chewing stimulated saliva than in the citric acid stimulated saliva (sign.). There was no significant difference in the concentration of CGRP between the resting saliva and the chewing stimulated saliva. There was seven-fold more CGRP in the chewing stimulated saliva than in the resting saliva released over time (sign.), but almost two-fold more in resting saliva than in the citric acid stimulated saliva (sign.). There was 10-fold more CGRP released over time in the chewing stimulated saliva than in the citric acid stimulated saliva (sign.).

Parotid saliva: There was a three-fold higher concentration of CGRP in the lowest flow as compared to the 0.50 mL/min flow of parotid saliva, but the difference was not significant. There was a ten-fold higher concentration of CGRP in the 0.25 mL/min flow than in the 1.00 mL/min flow (sign.) and three-fold higher concentration of CGRP in the 0.50 mL/min flow than in the 1.00
mL/min flow (sign.). There were no differences in the amounts of CGRP released over time in the three different flows of parotid saliva.

Neuropeptide Y (NPY)

Whole saliva: There was no significant difference in the concentration of NPY between the resting saliva and the chewing stimulated saliva, but there was three-fold more NPY released in the resting saliva than in the citric acid stimulated saliva (sign.) and 2.5-fold more in the chewing stimulated saliva than in the citric acid stimulated saliva (sign.). There was five-fold more NPY released over time in the chewing stimulated saliva than in the resting saliva (sign.), and three-fold more in the citric acid stimulated saliva than in the resting saliva (sign.). There was also two-fold more NPY released over time in the chewing stimulated saliva than in the citric acid stimulated saliva (sign.).

Parotid saliva: The concentration of NPY released in the 1.00 mL/min flow of the parotid saliva was almost 1.5-fold higher than in the 0.25 mL/min flow (sign.), but there were no significant differences between the 0.25 mL/min and the 0.50 mL/min flow, nor between the 0.50 mL/min and 1.00 mL/min flow. There was two-fold more NPY released over time into the 0.50 mL/min parotid saliva than into the 0.25 mL/min flow over time (sign.), and five-fold more into the 1.00 mL/min flow than into the 0.25 mL/min flow (sign.). Also, there was two-fold more NPY released over time into the 1.00 mL/min flow than into the 0.50 mL/min flow (sign.).

Vasoactive intestinal polypeptide (VIP)

Whole saliva: The concentration of VIP was 1.5-fold higher in the resting saliva than in the chewing stimulated saliva (sign.) and four-fold higher in the citric acid stimulated saliva (sign.). Also, the concentration of VIP was 2.5-fold higher in chewing stimulated saliva than in the citric acid stimulated saliva (sign.). There was three-fold more VIP released over time in the chewing stimulated saliva than in the resting saliva (sign.), two-fold more in the citric acid stimulated saliva than in the resting saliva (sign.), and two-fold more in the chewing stimulated saliva than in the citric acid stimulated saliva (sign.).

Parotid saliva: There were no significant differences in the concentration of VIP between the three different flows of parotid saliva. There was no significant difference in the amounts of VIP released over time between the 0.25 mL/min and the 0.50 mL/min flows of parotid saliva, but there was four-fold more VIP released over time in the 1.00 mL/min flow than in the 0.25 mL/min flow (sign). There was twice as much VIP in the 1.00 mL/min flow as in the 0.50 mL/min flow (sign).

There was a significant correlation between the volume of resting saliva and the release of CGRP and VIP (0.988 and 0.994 respectively). During chewing stimulation there was a significant correlation between the volume of produced saliva and the release of SP (0.929) and CGRP (0.994). During citric acid stimulation a strong correlation was found between volumes of produced saliva and the release of all investigated neuropeptides; SP (0.976), NKA (0.898), CGRP (0.826), NPY (0.952) and VIP (0.952).

Discussion

In our study we investigated five neuropeptides which are known to affect the salivary flow: SP, NKA, CGRP, NPY and VIP. They were collected under conditions similar to the natural events of saliva production, resting salivary flow, chewing and taste stimulation, as well as parotid
saliva which can be easily separated from the rest while its flow rates can be controlled precisely by taste stimulation. The results of this study showed that there were recurring patterns in the changes of the occurrence of these neuropeptides in the saliva produced under different conditions. These changes are easily discernible in Fig. 1. The actual contents of all five neuropeptides in the saliva of healthy subjects are summarised in TABLE I. To sum up the results of the entire study we were able to discern the following patterns of the changes in neuropeptide release into the saliva of healthy subjects:

The concentrations of the investigated neuropeptides in whole saliva decreased two- to fourfold (up to 16-fold for CGRP) when the volume of saliva increased 6 to 8 times due to stimulation. In the parotid saliva the concentration of CGRP decreased three- to ten-fold when the volume of saliva increased 2 to 4 times due to stronger stimulation, whereas the concentration of NKA increased three- to four-fold under the same circumstances. The concentrations of SP, and VIP did not change in the different flows of parotid saliva, while the concentration of NPY was significantly higher in the 1.00 mL/min flow as compared to the lower secretion rates. The release of neuropeptides changed also over time when the volumes of saliva produced/min increased. The amounts of all the investigated neuropeptides increased over time in the whole saliva two- to fivefold (up to ten-fold for CGRP) in the chewing-stimulated salivary flow as compared to the resting salivary flow or the citric acid stimulated flow. The amount of CGRP released over time was six- to ten-fold higher in the chewing stimulated saliva than in the resting saliva and citric acid stimulated saliva respectively. There was also two-fold more CGRP in the resting saliva than in the citric acid stimulated saliva. In the parotid saliva the amounts of all neuropeptides but CGRP released over time increased three- to 14-fold when the salivary secretion rates increased two- to four-fold, while the amount of CGRP did not alter at all.

The consistent decreases of the concentrations of all neuropeptides in the whole saliva as the salivary secretion rates increased indicate that the neuropeptides were released into the saliva in fairly constant amounts and diluted as the volumes of saliva increased. In the parotid saliva, however, the neuropeptides show a more inconsistent behaviour. The concentrations of SP and VIP did not change when the flow of saliva increased, whereas the concentration of NKA and NPY increased, and that of CGRP decreased, indicating that these neuropeptides are affected in a different manner by the stimulation itself. On the other hand, when the release of neuropeptides regarded as a function of time is analysed, new aspects surface. The amounts of all neuropeptides released over time increased when the salivary flow rates increase due to stimulation, indicating that the act of stimulation itself does indeed influence the release of neuropeptides from the nerve endings. It should be pointed out that CGRP increased spectacularly (16-fold) when the stimulation was caused by chewing (e.g. muscle work). Also, when citric acid stimulation was applied, which by itself caused a more lavish salivary flow, but resulted in even lesser release of CGRP than resting saliva. All neuropeptides showed more pronounced increases in the chewing stimulated saliva than in the citric acid stimulated saliva. These findings suggest that muscular action also causes an increase of the release of these neuropeptides, and are supported by several studies on the effect of exercise on neuropeptide release (33-38). In the parotid saliva we could observe that all neuropeptides but CGRP increased over time when the salivary flow rates were increased under controlled conditions due to citric acid stimulation (CGRP didn't show any changes). These findings support the notion that the release of neuropeptides can be increased by stimulation. The correlation found between the volumes of secreted saliva and the release of the neuropeptides seem to indicate that during rest both sensory and parasympathetic nervous systems are important for saliva secretion. During chewing the sensory system appears to play a more pronounced role. When citric acid stimulation is applied, an unspecified activation of both sensory and ANS nerve endings is induced. It should be pointed out that our results were probably also affected by the depletion of the neuropeptides as they are only stored in the nerve endings and a "shortage" might occur when they are "used up" due to lengthy stimulation. Also, although precautions have been
made in order to prevent the breakdown of neuropeptides during and after the saliva collection, some loss might occur. To diminish the influence of such factors, a highly standardised experimental protocol has been used.

It would be interesting to carry out a similar investigation on different groups of patients, especially those who suffer from salivary gland dysfunction, as well as under therapeutic conditions.

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