Effect of electrical tooth stimulation on blood flow and immunocompetent cells in rat dental pulp after sympathectomy

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Previous experiments show that nerves have effect on the emigration of immunocompetent cells during acute neurogenic inflammation. The present study aims to determine whether the sympathetic or sensory nerves are responsible for emigration of CD43+ and I-A antigen-expressing cells in the dental pulp after electrical tooth stimulation. Wistar rats were used. Experimental rats ($n = 6$) had the right superior cervical ganglion removed (SCGx), whereas control rats ($n = 6$) had sham surgery. Fourteen days later, electrical stimulation of the right maxillary 1st molar was performed in both groups for 20–25 s every 5th min for a total period of 4 h. Changes in pulpal blood flow (PBF) were recorded with a laser Doppler flowmeter. All rats were transcardiacally perfused and processed for immunohistochemistry using antibodies against neuropeptides and immune cells. Intermittent electrical stimulation consistently increased PBF and depleted sympathetic and sensory neuropeptides in the dental pulp. The increase in PBF gradually decreased and approached control values at the end of the 4 h stimulation period. A significant increase in the number of I-A antigen-expressing dendritic cells was found in both the SCGx ($P < 0.001$) and control rats ($P < 0.007$). In contrast, tooth stimulation did not increase the number of CD43+ cells in the SCGx rats compared to the unstimulated contralateral control molar. Significantly more CD43+ PMN cells ($P < 0.01$) were found in the control rats after stimulation. It is concluded that stimulation of sympathetic nerves causes recruitment of CD43+ PMN cells, whereas stimulation of sensory nerves causes emigration of I-A antigen-expressing dendritic cells in the dental pulp. □ *CD43+ cells; I-A antigen-presenting cells; sensory nerves; sympathetic nerves*

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Current evidence suggests that the sympathetic nerves play a significant role in inflammation and immune responses. It has been shown that sympathetic denervation results in loss of regulatory mechanisms in the immune system and changes the migratory ability of lymphocytes (1–3). The dental pulp is well equipped with immunocompetent cells essential for immunological responses (4–6) and close anatomical connections between the immunocompetent cells and nerves have been identified (7–9). Recently, we found that unilateral removal of the superior cervical ganglion (SCG) induced an increase in neuropeptideY-immunoreactive (NPY-IR) fibers contralateral to the sympathectomy and an increase in the number of large unidentified immunocompetent cells in the dental pulp (7). Furthermore, previous experiments show that the nerves in the dental pulp participate in and facilitate emigration of immunocompetent cells during acute neurogenic inflammation induced by electrical tooth stimulation (10). In our previous experiments, no recruitment of immune cells was observed after inferior alveolar nerve (IAN) axotomy, by which both sensory and sympathetic nerves are sectioned (11, 12). The present study was therefore aimed at identifying a specific role for the sympathetic nerves on immune cell recruitment in the dental pulp. We test whether the sympathetic or sensory nerves are responsible for the increased number of CD43+ and I-A antigen-expressing cells in the dental pulp after long-lasting intermittent electrical tooth stimulation.

Materials and methods

Experiment procedure and animals

Female Mol:Wistar rats of average body weight 152 g (range 117–76) were used, and the animal experiments were approved by the Norwegian Experimental Animal Board.

The animals were anesthetized with 0.16 mL/kg Hypnorm-Dormicum (1 mL fentanyl/fluanison diluted in 1 mL sterile water mixed with 1 mL midazolam diluted in 1 mL sterile water) injected subcutaneously. A vertical incision was made on the ventral surface of the neck adjacent to the midline. The right superior cervical sympathetic ganglion (SCG) was identified using a stereomicroscope and thereafter surgically removed (SCGx). Successful removal was confirmed by observation of Claude Bernard-Horner's syndrome on the operated side (ptosis of the eyelid). Sham-operated rats served as control. Sham surgery involved anesthesia, incision and

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localization of the SCG. The incision site was thereafter sutured. All rats regained normal weight and feeding habits.

Fourteen days after sympathectomy, all rats were intraperitonally anesthetized with Mebumal (sodium pentobarbital, 50 mg/kg, Svaneapoteket, Bergen, Norway). The animals were intubated to provide free respiration and placed on a heating pad, thermostatically controlled to maintain rectal temperature at 37°C. A femoral vein was catheterized for supplemental anesthesia (0.2 mL Mebumal, diluted 1:5 in saline) when needed. A femoral artery was catheterized for continuous systemic arterial blood pressure (PA) recordings. The head was immobilized and simultaneous measurements of PA and pulpal blood flow (PBF) were continuously performed throughout the experiments.

Pulpal blood flow recordings

Changes in PBF were measured with a laser Doppler flowmeter (LDF) (PeriFlux, Model 4001; Perimed AB, Järfalla, Sweden) equipped with a probe with fiber diameter 125 μm. The probe was fixed in a micromanipulator and placed on the mesial surface of the right maxillary 1st molar in the position which gave the largest blood flow signal. Motility standard and flux zero setting of the flowmeter were set in accordance with the manufacturer. Zero value was determined as the value recorded from a stationary white card.

As the LDF measures only relative changes in the flux of blood cells times the velocity, changes in the LDF recordings obtained during electrical stimulation were calculated as percentages of the baseline values during control conditions.

Electrical tooth stimulation

Electrical stimulation of the right maxillary 1st molar was performed in both SCGx and nonSCGx control rats. After acid etch for 40 s (Scotchbond 3 M Dental Products, St. Paul, Minn., USA), followed by water rinse and air drying, an electrode was placed in the mesial fissure of the 1st molar and fixed into position with a flowable composite resin (Tetric Flow; Ivoclar Vivadent AG, Schaan, Lichtenstein). The indifferent electrode was placed in the ipsilateral cheek. Before stimulation, a resting period of at least 30 min was provided to achieve steady-state blood flow values. A constant voltage unit (Grass Co., S44, Quency, Mass., USA) giving square-wave pulses (2 ms, frequency 1–20 Hz and voltage 10–25) was used for pulp stimulation for 20–25 s every 5th min for a total period of 4 h.

At the end of the experiments, all rats were deeply anesthetized and transcardiacally perfused with heparinized saline, followed by 4% paraformaldehyde with 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The jaws were excised, postfixed and demineralized in 10% EDTA. After cryoprotection with 30% sucrose, the jaws were stored at −80°C until sectioning.

Immunohistochemistry

Alternate serial cryostat sections (35 μm) of the jaws from the experimental and control group were processed for immunohistochemistry on pre-coated glass slides (SuperFrost Plus; Menzel-Glaser, Braunschweig, Germany) with one of the following primary antibodies (Table 1). The monoclonal antibodies Ox-6 (1:3000, Seralab, UK) and W3/13 (1:2000, Seralab, UK) were used for staining of I-A antigen-expressing and CD43+ cells, respectively. Sections were rinsed in phosphate buffered saline (PBS) and treated for 30 min with 0.3% hydrogen peroxide in absolute methanol. Thereafter the sections were presoaked for 3 h in blocking serum, a combination of 2.5% normal horse (Harlan Sera-Lab Ltd., UK) and rat serum (Chemicon Intl. Inc., Temecula, USA). The primary antibodies were incubated with blocking serum overnight at 4°C. After several rinses, sections were incubated overnight in biotinylated anti-mouse immunoglobulin G (1:100 dilution) (Vector Laboratories Inc., USA) with blocking serum. Following several PBS rinses, sections were incubated in ABC reagent (Elite ABC kit, Vector) for 4 h.

For staining of sympathetic nerve fibers, alternate serial sections were incubated for 72 h in a polyclonal NPY antibody (rabbit IgG, 1: 4000 dilution) (Peninsula Laboratories, USA) and for sensory nerve fibers in a polyclonal calcitonin gene-related peptide (CGRP) antibody (rabbit IgG, 1:6000 dilution) (Peninsula Laboratories, USA). The sections were rinsed in PBS and treated with 0.3% hydrogen peroxide in absolute methanol. Sections were next incubated in 2.5% normal goat serum (Vector) for 1 h. Primary antibodies were incubated with 2.5% normal goat serum for 72 h at 4°C. After several rinses in PBS, sections were incubated for 1 h in biotinylated anti-rabbit immunoglobulin G (1:1000 dilution) (Vector). Following several PBS rinses, sections were incubated in ABC reagent (Vector) for 1 h. Final visualization for all antibodies was made with nickel-enhanced 0.025% 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Inc., St. Louis, Minn., USA) with H_2O_2 as chromogen, counterstained in Richardson's stain, and coverslipped with Assistent Histokitt (Germany). Controls of the specificity of the immunoreactions were routinely performed by substitution of the primary or secondary antiserum with PBS.

Table 1. Primary antibodies for immunohistochemistry

Antibody	Antigen expression	Reference
Ox-6	I-A antigen present on B-lymphocytes and dendritic cells	(27)
W3/13	CD43+ T lymphocytes, granulocytes and plasma cells	(28)
Rat NPY	NPY in neuronal cell bodies and axons	(29)
Rat CGRP	CGRP in neuronal cell bodies and axons	(30)

Quantification and statistics

Eight central longitudinal sections (4 sections immunoreacted for Ox-6 and W3/13, respectively) from stimulated and contralateral unstimulated 1st molars from 6 SCGx and 6 control rats were used for quantification of immunocompetent cells by 2 different methods. The sections were evaluated using a computer program and also counted in a light microscope. The immunoreactive cells were counted blindly in a grid system, total area 1×10^4 μm^2, in the pulp subjacent to the insertion site of the electrode, as previously described (10). I-A antigen-expressing and CD43+ cells were also quantified using a Nikon Dxm 1200 digital camera and a Nikon E600 microscope connected to an image analysis program (Lucia G version 4.71; Laboratory Imaging). Digital images (2×10^4 μm^2) in the pulp subjacent to the stimulating electrode were collected and subjected to a normalization procedure in order to equalize the background levels across sections. Subsequently, the fields were quantified per image, by measuring the area in μm^2 of the IR cells.The Wilcoxon signed-rank test was used for testing of difference between paired data. For unpaired data, the Mann-Whitney test was applied. Data are given as mean ± $s_{\bar{x}}$ (standard error of the mean).

Results

Laser Doppler flow (LDF) recordings

Simultaneous recordings of PBF and PA were performed continuously before, during, and after electrical stimulation of the 1st right maxillary molar in SCGx and control rats for up to 4 h. Provided unchanged position of the probe, the baseline value in control measurements remained stable throughout the individual experiments. In accordance with previous measurements (10), intermittent electrical stimulation every 5th min consistently increased PBF, whereas PA remained unchanged. Average PA in the 12 rats was 106 ± 6 mmHg. The increase in PBF following stimulation gradually decreased and approached control values at the end of the 4 h stimulation period.

There was no significant difference in laser Doppler output between the 2 groups, neither in control measurements nor during electrical tooth stimulation for 4 h (Fig. 1).

Average increase in PBF in control animals during the first 15 min of stimulation was 33 ± 5% compared to 34 ± 4% in the sympathectomized animals (Fig. 1).

Immunohistochemistry

Nerve fibers. The stimulated 1st molar in the control group showed an almost total loss of NPY- and CGRP-IR fibers (Figs 2 and 3) 4 h after intermittent electrical stimulation, whereas the ipsilateral 2nd and 3rd molars showed no difference in nerve fiber immunoreactivity when compared with the contralateral controls. The depletion of CGRP- and NPY-IR nerve fibers shows that electrical tooth stimulation causes release of these neuropeptides from the peripheral nerve endings.

Immunocompetent cells. Electrical stimulation increased the number of I-A antigen-expressing cells in the 1st molar coronal pulp (Fig. 4). The I-A antigen-expressing cells were identified as dendritic cells with cytoplasmic extensions (Fig. 4). Compared to the contralateral non-stimulated 1st

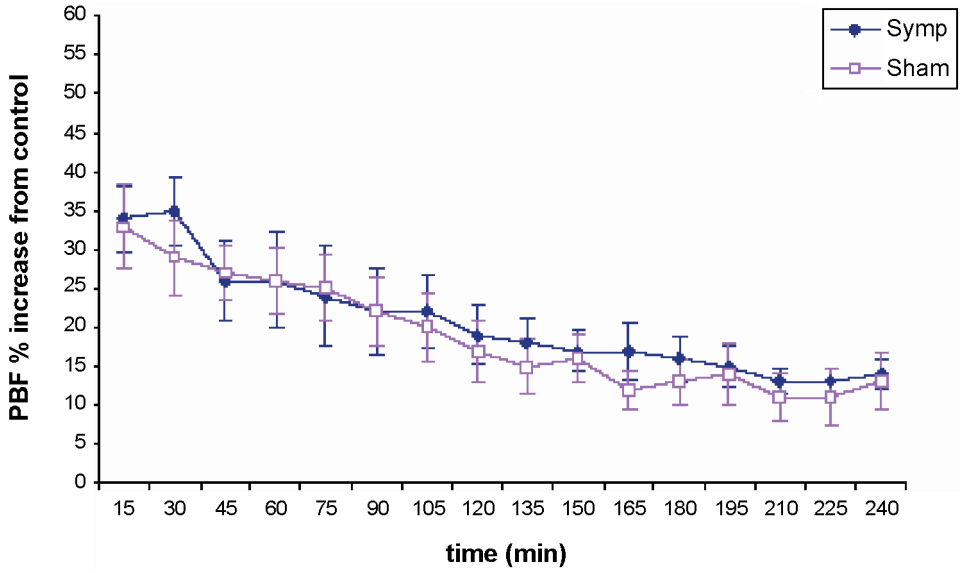

Fig. 1. Graph showing effect of repeated electrical tooth stimulation versus time on percentage increase in pulpal blood flow (PBF). Values represent mean of three stimulation periods ± $s_{\bar{x}}$. Filled symbols are sympathectomized ($n = 6$) and open symbols sham-operated control ($n = 6$) rats.

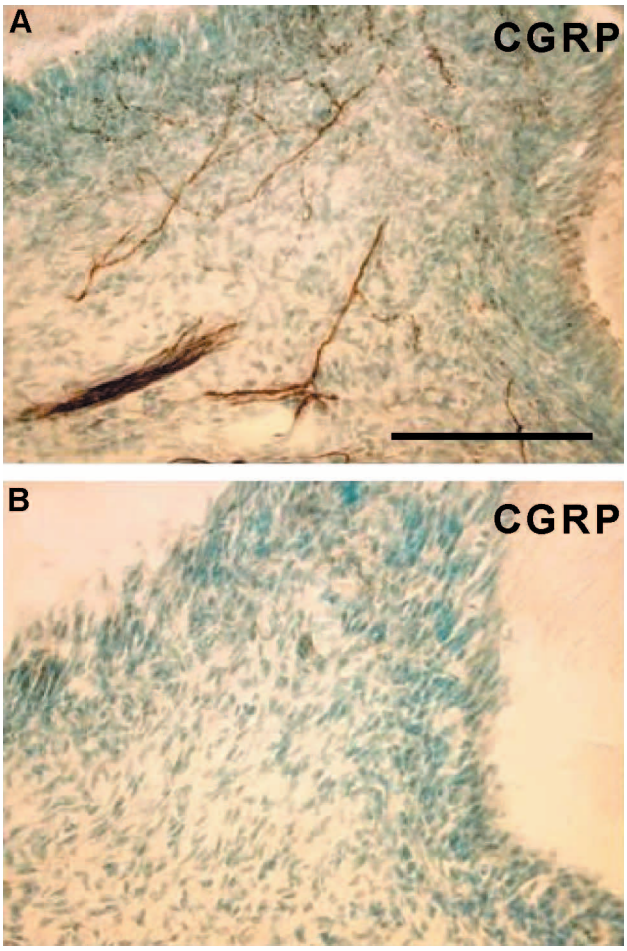

Fig. 2. Immunohistochemical micrographs from 1st molar pulp of a control rat. A. Presence of CGRP immunoreactive fibers in non-stimulated 1st molar pulp (left side). B. Absence of CGRP nerve fibers in contralateral 1st molar pulp after electrical tooth stimulation (right side). Bar = 100 μm.

molar, significantly more IR cells were found in both the SCGx ($P < 0.001$) and control rats ($P < 0.007$) after stimulation (Fig. 5A, B). There was no significant difference in the I-A antigen-expressing cell number between the two groups, indicating that sympathetic denervation had no effect on emigration of these cells.

In contrast, electrical tooth stimulation did not increase the number of CD43+ cells in the SCGx rats compared to the unstimulated contralateral control molar, whereas a significantly higher number of CD43+ cells ($P < 0.05$) was found in the innervated control rats after stimulation (Figs 6 and 7). Morphologically, the majority of the CD43+ cells were round, with an average diameter of approximately 8 μm. Based on morphology and staining characteristics, these cells were identified as polymorphonuclear (PMN) (Fig. 6).

Discussion

The present study was designed to identify a potential role of the sympathetic nerves in immune cell recruitment in the rat molar pulp in vivo. Our experiments demonstrate that the sympathetic as well as the sensory nerves facilitate transendothelial emigration of white blood cells. Previous studies have analyzed the participation of dental nerves in immunocompetent cell recruitment and inflammation in the dental pulp after tooth denervation by IAN axotomy (13, 14). In studies employing IAN axotomy, it was concluded that the responses were solely due to loss of sensory nerves, while no attention was paid to the loss of sympathetic fibers. Thus the arrival of immune cells into injured pulp has primarily been assigned to the sensory nerves. Similarly, expanded areas of pulpal necrosis after

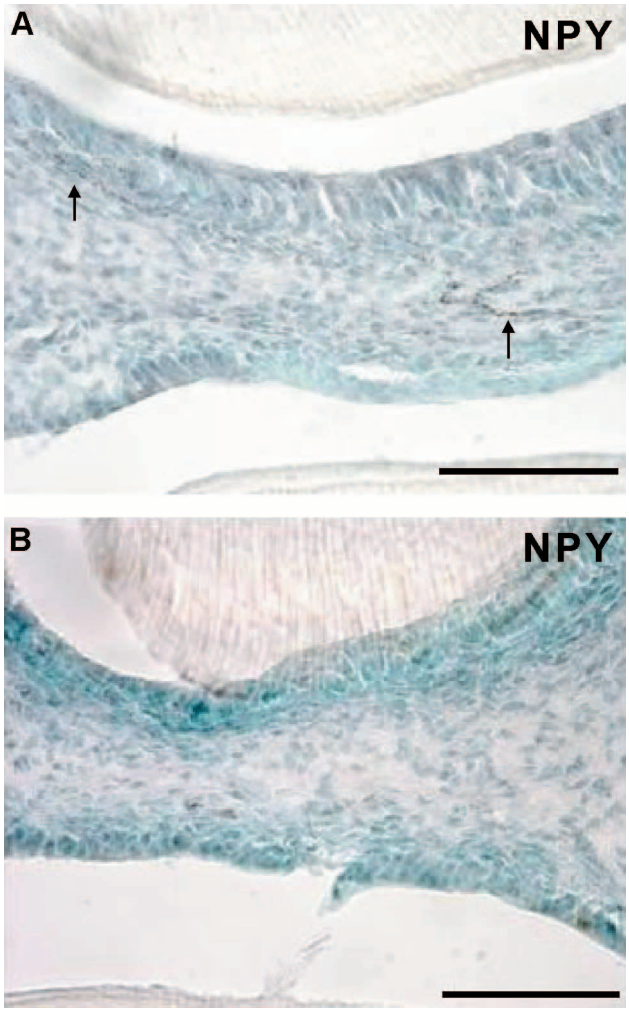

Fig. 3. Immunohistochemical micrographs from 1st molar pulp of a control rat. A. Presence of NPY immunoreactive fibers (arrows) in non-stimulated 1st molar pulp (left side). B. Absence of NPY IR nerve fibers in contralateral 1st molar pulp after electrical tooth stimulation. Bar = 100 μm.

IAN axotomy have been attributed to the lack of sensory nerves (14). However, not only is the sensory nerve supply to the dental pulp eliminated by IAN axotomy, but also the major portion of the sympathetic innervation (11, 12).

The present experiments show, for the first time, that electrical stimulation of sympathetic nerves causes recruitment of immune cells in the dental pulp. In teeth lacking sympathetic nerves, electrical stimulation did not increase the number of CD43+ cells, whereas stimulation of control rats with an intact sympathetic nerve supply caused a significantly higher number of these cells compared to the contralateral non-stimulated dental pulp. This is in line with earlier findings (10), where we found a significantly higher number of CD43+ cells in innervated, but not in pulps denervated, by IAN axotomy. As selective sympathetic denervation gives the same results as IAN denervation, the present findings indicate that it is the sympathetic nerves, and not the sensory nerves, that promote recruitment of CD43+ cells in the dental pulp after electrical tooth stimulation.

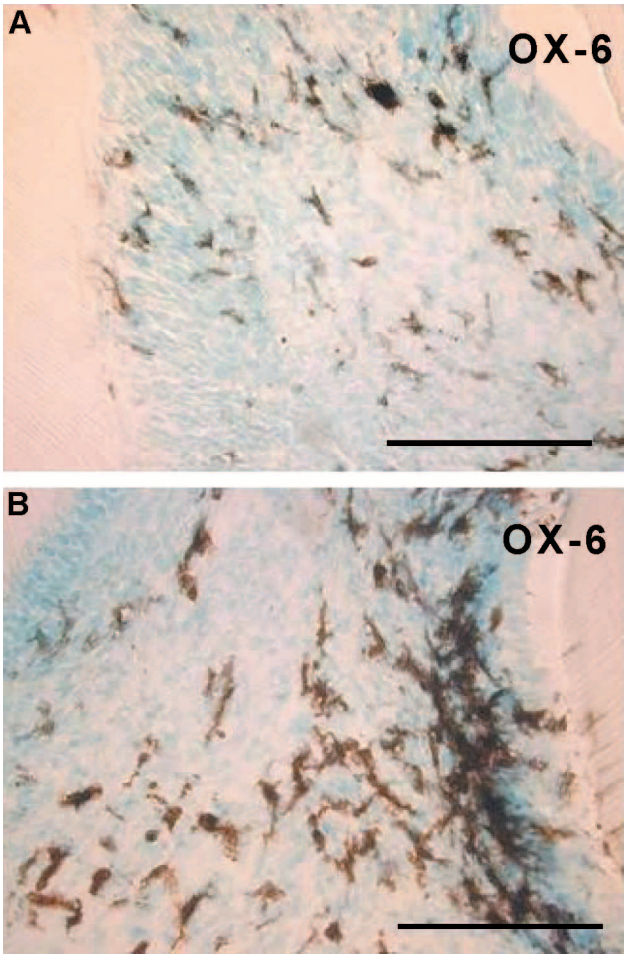

Fig. 4. Immunohistochemical micrographs from a unilateral sympathectomized rat showing I-A antigen-expressing (Ox-6 IR) cells in maxillary 1st molar pulp. A. Few I-A antigen-expressing cells in unstimulated pulp. B. Increased number of cells in contralateral pulp after 4 h of electrical tooth stimulation. Bar = 100 μm.

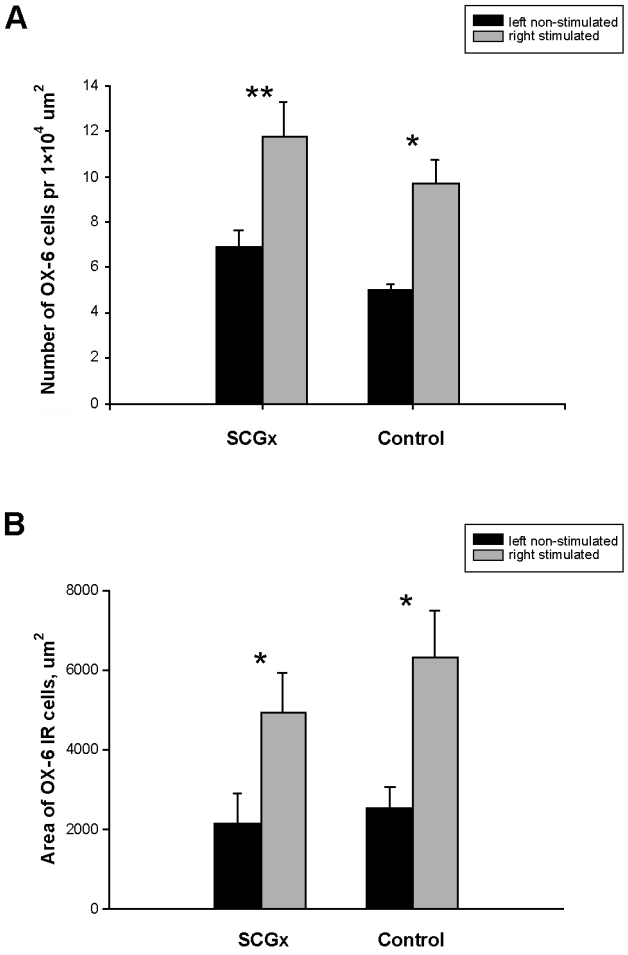

Fig. 5. I-A antigen-expressing (Ox-6 IR) cells in the dental pulp. A. Number of cells counted. B. area of cells as quantified by image analysis. Effect of 4 h intermittent electrical tooth stimulation in sham-operated controls ($n = 6$) and in unilateral sympathectomized (SCGx) rats ($n = 6$). Values represent mean ± $s_{\bar{x}}$; * $P < 0.007$, ** $P < 0.001$.

In agreement with previous experiments (10), stimulation increased the number of I-A antigen-expressing cells in both groups, suggesting that the sympathetic nerves have little effect on recruitment of these cells. In the normal pulp of rats and humans, I-A antigen-expressing cells, with class II molecules on their surface, are a common feature (6, 15). The location of class II molecule-expressing cells is mainly perivascular as well as in the para-odontoblastic regions. The latter location suggests that class II molecule-expressing cells are organized at sites of potential antigen challenge and may interact as a barrier against invasion of antigenic stimuli to the pulp from the oral environment. The dense network of sensory nerves in the para-odontoblastic regions might advocate a functional significance between nerves and immunocom-

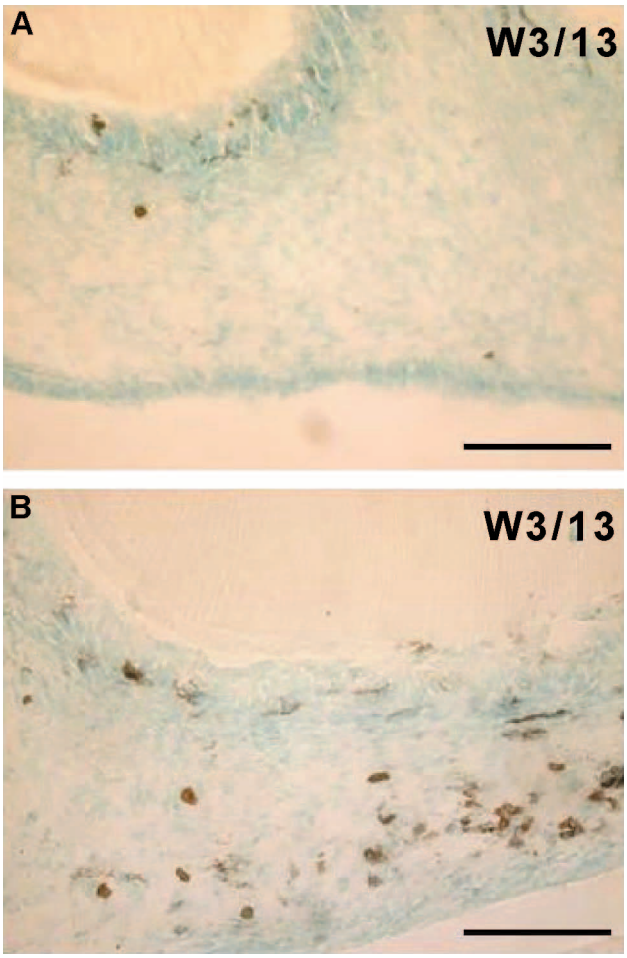

Fig. 6. Immunohistochemical micrographs from a control rat showing CD43+ (W3/13 IR) cells in maxillary 1st molar pulps. A. Very few cells in non-stimulated 1st molar pulp. B. Increased number of cells in contralateral 1st molar pulp after 4 h of electrical tooth stimulation. Bar = 100 μm.

petent cells. In addition, the nerves in the para-odontoblastic regions are shown to sprout in response to inflammation (16). The observation of Okiji et al. (9), that I-A antigen-expressing cells in the rat molar pulp frequently exhibited contact to thin, varicose nerve fibers IR to SP and/or CGRP, shows the presence of morphological neuro-immune connections in the pulp. However, until now, little information is available regarding a functional significance between nerves and immunocompetent cells in the dental pulp in vivo. The pronounced reduction of NPY- and CGRP- IR fibers after electrical stimulation in nonSCGx rats indicates that these neuropeptides are released from the nerve terminals during the present experimental procedure. It therefore seems reasonable to assume that the released neuropeptides may act, either directly or indirectly, to recruit immune cells in the dental pulp. Immune cell recruitment is traditionally thought to be mediated by cytokines (17). Earlier studies indicate a direct linkage between neurogenic stimulation and proinflammatory cytokine release (18, 19). In vitro studies of bone marrow cells show that CGRP induces production of interleukin-6 and TNF-alpha (20), whereas substance P increases interleukin-8 production in human dental pulp cells (21). Taken together, these findings suggest that neuropeptides released from the nerve terminals may play a role in immune cell recruitment indirectly by affecting the cytokine production.

Stimulation of sensory nerves is well known to cause neurogenic inflammation, unrelated to infection. However, recent findings strongly indicate that the sympathetic nerves, too, play a significant role in inflammation and immune responses. In contrast to the I-A antigen-expressing cells, the occurrence of CD43+ cells is scarce

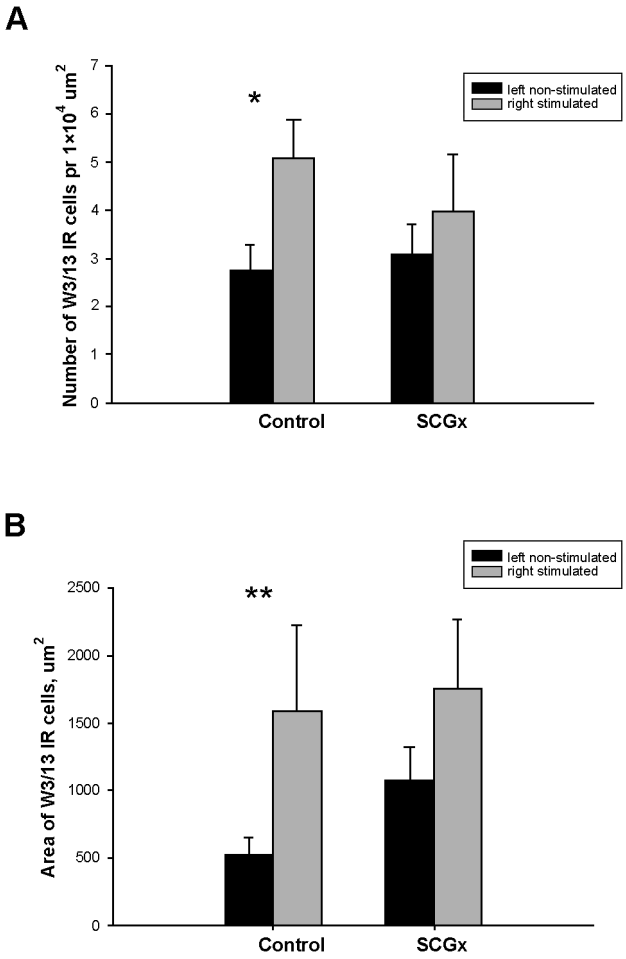

Fig. 7. CD43+ (W3/13 IR) cells in the dental pulp after 4 h of intermittent electrical stimulation in sham-operated controls ($n = 6$) and in unilateral sympathectomized (SCGx) rats ($n = 6$). A. Number of counted cells. B. Area of cells as quantified by image analysis ($n = 6$). Values represent mean ± $s_{\bar{x}}$; * $P < 0.05$, ** $P < 0.01$, stimulated compared to contralateral non-stimulated teeth.

in the normal uninflamed dental pulp, whereas they are frequently found during inflammation (22). However, CD43+ plasma cells and CD4+ lymphocytes are not a common finding under normal conditions or during acute neurogenic inflammation (10, 13). As PMN cells represent the first line of defense, it is reasonable that the majority of the CD43+ cells in the present acute experiments represent PMN cells. Our results are in agreement with a previous study suggesting that sympathetic nerves are responsible for the recruitment of CD43+ cells during uninfected inflammation induced by orthodontic tooth movement (23).

The intra-individual differences between stimulated and non-stimulated control teeth were significant both by cell counting and by image analysis. One might, however, expect to find more W3/13 IR cells in stimulated control teeth when compared to SCGx teeth after stimulation. However, both cell quantification methods were in agreement, indicating that the lack of difference between control and experimental rats after stimulation might be explained by interindividual differences. The relatively high basal level of W3/13 in non-stimulated teeth in SCGx rats is most readily explained by the increase in sympathetic nerve density that is shown to occur contralateral to the sympathectomy (7).

We found no significant difference between the present blood flow measurements in SCGx and control rats, neither during control conditions nor after electrical tooth stimulation. The observation during control conditions agrees fairly well with previous experiments in dogs, suggesting nervous sympathetic vascular basal tone to be absent in the pulp (12). However, during stimulation it might be expected that the SCGx pulps, lacking sympathetic vasoconstriction fibers, would give higher blood flow rates than the control rats. The explanation might be that the vasodilatation caused by release of sensory neuropeptides overwhelms the vasoconstriction induced by sympathetic stimulation, thereby masking the sympathetic vasoconstrictor response. Earlier studies have shown that about 90% of the total number of nerves in teeth is of sensory origin (24–26), and the release of neuropeptides after electrical stimulation might be expected to be proportional to the number of nerves. On the other hand, the laser Doppler output is not an absolute value, and comparisons between groups are therefore complicated.

In conclusion, the present results provide evidence that stimulation of sympathetic nerves causes recruitment of CD43+ PMN cells, and that stimulation of sensory nerves causes emigration of I-A antigen-expressing dendritic cells in the dental pulp of rats.

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