

## INVESTIGATIVE REPORT

# Cutaneous Field Stimulation with Moderate Intensity Current Induces Nerve Proliferation in Rat Skin But Has No Effect on Dorsal Root Ganglia

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Cutaneous field stimulation is used to treat localized itch. The aim of the present study was to determine whether such treatment induces neurochemical changes in the dorsal root ganglia in 30 rats using a pan-neuronal marker protein gene-product 9.5 (PGP 9.5) and calcitonin gene-related peptide (CGRP). Electrical stimulation using the currents of either 0.13 mA or 0.53 mA - was given under general anaesthesia for 30 min per day for 10 days. Punch biopsies from the thoracal skin and the corresponding dorsal root ganglia were collected upon sacrifice. Both stimulation regimens induced proliferation of epidermal and dermal nerve fibers in the skin. The mean number of all cutaneous PGP-immunoreactive (IR) nerve fibers after the electrical stimulation with 0.13 mA was increased by 49% ( $p < 0.001$ ), the mean number of epidermal PGP-IR nerve fibers was increased by 25% ( $p = 0.001$ ) and the mean number of all CGRP-IR nerve fibers was increased by 65% ( $p < 0.001$ ) compared with controls. The mean number of all PGP-IR nerve fibers after the electrical stimulation with 0.53 mA was increased by 39% ( $p < 0.001$ ), the mean number of PGP-IR epidermal nerve fibers was increased by 30% ( $p = 0.001$ ) and the mean number of CGRP-IR nerve fibers was increased by 65% ( $p < 0.001$ ) compared with controls. Only stimulation with 0.53 mA induced an up-regulation of sensory neuron markers in the dorsal root ganglia. The ratio of positive/negative PGP-IR cells was increased by 17% ( $p = 0.002$ ), the ratio of positive/negative CGRP-IR cells was increased by 12% ( $p = 0.003$ ) and the ratio of positive/negative VR1-IR cells was likewise increased by 10% ( $p = 0.008$ ) as compared with the control ganglia. We conclude that serial cutaneous electrical stimulation by a moderate current in rat does not induce neurochemical changes in the dorsal root ganglia. *Key words: itch; nerve fibers; dorsal root ganglia; CGRP; PGP 9.5; vanilloid receptor.*

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Scratching, rubbing, massage and even painful stimulation of the skin are basic approaches people use for

relieving itch. In 1965, Melzack & Wall (1) presented a new theory of pain. This theory, since modified, postulates that the stimulation of large diameter nerve fibers impairs nociceptive transmission by activating inhibitory neurons of the spinal cord. On the basis of these findings, transcutaneous nerve stimulation (TENS) has been developed for the activation of large myelinated nerve fibers (2–4). A low-frequency TENS (2 Hz), shown to reduce itch, employs a stimulator generating an alternating current (5, 6). The sensation of itch is mediated by a specific subset of epidermal C-fibers that are insensitive to mechanical stimuli but sensitive to thermal stimuli and comprise about 5% of the C-fibers (7). A new technique, called cutaneous field stimulation (CFS), which activates unmyelinated C-fibers electrically, was developed by Nilsson et al. (8) for treating localized itch. The CFS technique differs from TENS: (i) in the construction of the cathode, (ii) in the anode being placed close to the treated area, and (iii) by the type of stimulator employed.

The peak inhibitory effect of CFS treatment for 25 min in patients with chronic itch was found to be between 1 and 5 h post-conditioning (9). When patients with localized itch were treated by CFS for 30 min once a day for 5 weeks, itch diminished from 78% before treatment to 42% by the end of the fifth week as measured on a visual analogue scale (10). In skin biopsy specimens collected before and after treatment, a 40% reduction in epidermal innervation was shown using the pan-neuronal marker protein gene-product 9.5 (PGP) (10). Such local changes in cutaneous innervation would suggest the involvement of peripheral mechanisms in the CFS effects.

Central mechanisms, with CFS activating the spinal cord and exerting segmental effects on the distribution of a dermatome, have also been suggested (9). The finding of local inhibition of itch up to a distance of 10 cm distal from the CFS electrode and greatly exceeding the duration of a single CFS has been put forward as an argument for a central effect. The aim of the present experimental study was to determine whether CFS treatment induces morphological and neurochemical changes in the dorsal root ganglia (DRG) of rats as an indication of a central effect.

## MATERIALS AND METHODS

### *Animals*

A total of 30 male rats (Sprague-Dawley), 8 weeks of age, weighing approximately 250 g, were included in the study. The animals were housed in groups of three, with food and water available ad libitum, and were maintained under a light/dark cycle (12 h light and 12 h dark) for about a week prior to the experiments.

### *Cutaneous field stimulation device*

The CFS device employed consists of a cathode (a flexible rubber electrode plate, 8 × 8 cm in size), an anode (a flat reference electrode, 5 × 5 cm) placed close to the treated area and a stimulator (9 V) (8). The CFS electrode plate is covered with 16 needle-like electrodes (0.3 mm in diameter) surrounded by a 'sop-device' protruding 2 mm from the plate. The plate was gently pressed onto the area of skin to be treated, the electrode tips being positioned in the epidermis and the superficial layer of the dermis. A constant current with monophasic square pulses, a 1-ms duration, a 4-Hz frequency and a current intensity ranging from 0.03 to 1 mA is delivered to each electrode.

### *Animals treated with cutaneous field stimulation*

Two experimental groups each consisting of nine rats were subjected to two different regimens of percutaneous electrical stimulation with a CFS device. The stimulation was administered for 30 min per day for 5 days. The animals were then allowed to rest for 2 days, after which they were subjected to daily stimulation for another 5 days. All treatments were performed under general anaesthesia using 10 mg ketamine hydrochloride (Ketamine®) and 0.1 mg medetomidine hydrochloride injected intraperitoneally (i.p.).

The skin of the back and of the central part on the abdomen was shaved to allow the electrodes to be applied. The cathode was placed on the dorsal side, covering the area extending from the lower part of the cervical column to the lumbar section of the spinal column, the reference electrode being placed on the abdominal skin. The rats belonging to the first experimental group were stimulated on the first day by a 0.04-mA current for 30 min, and from the second day onwards by a 0.13-mA current for 30 min per day. The rats in the second experimental group were stimulated by a 0.27-mA current for 30 min during the first day, by a 0.40-mA current on the second day and thereafter by a 0.53-mA current for 30 min per day. Between 2 and 3 h after the final treatment, the rats were killed by a lethal injection of 500 mg pentobarbital i.p. Thereafter, skin punch biopsies (5 mm diameter) were taken using a punch device (Kai Medical, Seki City, Japan) from the skin covering ribs 11–13 on the side of the column and all DRG at level Th12–13 on both sides were dissected by careful excision of the dorsal roots. In six of the control animals, as well as in the first experimental group, two skin biopsies from the thoracal skin on each side of the column were collected (dermatomes Th12–13), whereas in the remainder of the control animals and in the second experimental group only a single biopsy was obtained.

The study was approved by the regional local animal ethics committee.

### *Control animals*

Twelve rats served as controls. These animals rested in their respective cages for 2 weeks and were not subjected to

anaesthesia or shaving. Skin biopsies and spinal DRG were collected following sacrifice of the animals.

### *Processing of biopsies*

The specimens were fixed by immersion overnight in a mixture of 2% formaldehyde and 0.2% picric acid solution in a 0.1 mol/l phosphate buffer (pH 7.2) and were then thoroughly rinsed in a Tyrode solution containing 10% sucrose. They were frozen thereafter on dry ice and serially sectioned in 10- $\mu$ m slices on a cryostat. The sections were then processed for indirect immunofluorescence, using polyclonal antibodies against the neuropeptide calcitonin gene-related peptide (CGRP) and a pan-neuronal marker, PGP 9.5. Antibodies against CGRP (Euro Diagnostica, Malmö, Sweden; working dilution 1:1200) were used to demonstrate the sensory C-fibres. The PGP 9.5 antibodies (Ultraclone, Cambridge, UK; working dilution 1:200) were used as marker for the visualization of all cutaneous nerve fibres. PGP 9.5 is a cytoplasmic constituent in all parts of the neurons, the cell bodies as well as all processes. Data on the specificity of the antibodies employed have previously been presented (11). Polyclonal antibodies against the vanilloid receptor, VR1 (Euro Diagnostica; working dilution 1:640), were used for the identification of capsaicin-sensitive nociceptive primary afferents (12). The vanilloid, capsaicin, the primary agent contained in hot pepper, is known to trigger the release of neuropeptides from sensory nerve fibers (13). FITC-labelled swine-anti-rabbit Ig G antibodies (DAKO Copenhagen, Denmark; dilution 1:40) served as secondary antibodies. Controls in the immunohistochemical procedure included omission of the primary antibody and/or preabsorption of the antibody with antigen in excess (100  $\mu$ g peptide/ml antibody at working dilution).

Three consecutive sections of each of the biopsies were studied. The microscope used for the analysis was a Leica Aristoplan (epifluorescence microscope). Coded sections were evaluated by a single observer. The number of immunoreactive (IR) nerve fibres in the epidermis and the dermis was assessed visually at  $\times 250$  magnification. The number of IR nerve cell bodies in the ganglia was counted at the same magnification; only cells with a visible nucleus were counted. All IR nerve fibre segments in the biopsy section as a whole were counted, even if a single undulating and branching nerve fibre may appear more than once. Micrographs were taken using Kodak EPL 400X film.

### *Statistical analysis*

The statistical evaluation is based on the means of the counts made in the three sections for each biopsy. The means of two biopsies and the means of all ganglia taken from each side were calculated. The results are expressed as mean  $\pm$  SEM. Student's paired *t*-test was used for comparing the skin biopsies obtained after treatment by CFS with those of the controls.

## RESULTS

### *General characteristics of the experimental animals*

The control rats gained weight from  $261 \pm 9$  g at the beginning of the experiment to  $326 \pm 16$  g at the end of the experiment 11 days later ( $p < 0.001$ ). Percutaneous stimulation in CFS group I (0.13 mA current for 30 min) produced no visible muscle contractions. The rats in this CFS group gained weight from  $255 \pm 8$  to

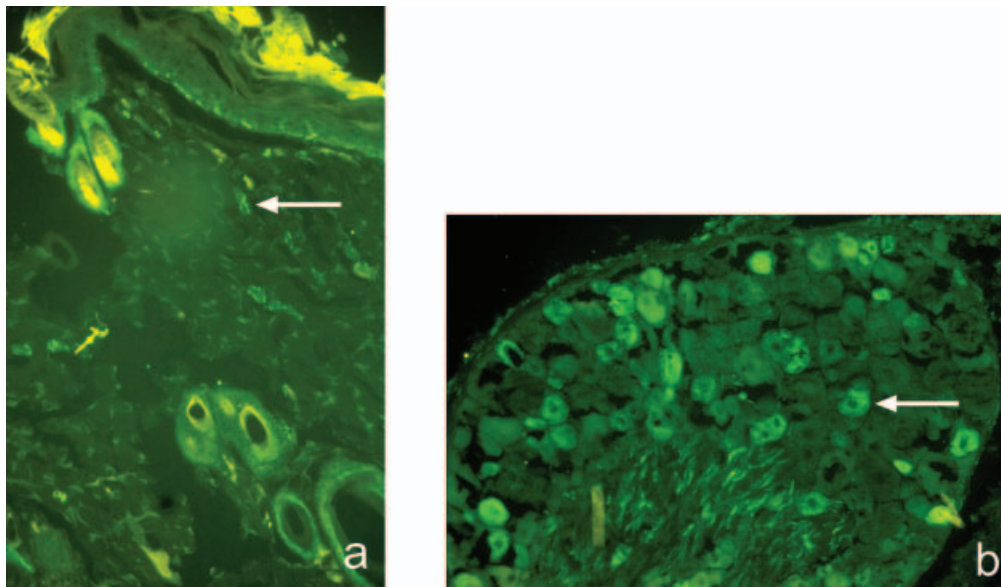


Fig. 1. Skin biopsies of control rats showing PGP 9.5-immunoreactivity (IR) (a) and CGRP-IR (b) in nerve fibers (arrows). Magnification  $\times 250$ .

$282 \pm 1$  g ( $p=0.004$ ). Percutaneous stimulations in CFS group II (0.53 mA for 30 min) produced visible muscle contractions. The rats appeared stressed, sometimes biting each other, and did not gain weight ( $262 \pm 11$  g vs  $255 \pm 10$  g,  $p=0.20$ ).

**Control group.** The skin on the flank was richly innervated by PGP 9.5-IR nerve fibers, whereas the density of CGRP-IR nerve fibers was moderate (Fig. 1). The nerve fibres in the skin did not display any VR1-IR. The mean number of PGP 9.5-IR nerve fibres in the epidermis was 108 per biopsy section, while the total number of cutaneous PGP 9.5-IR nerve fibres was 209 per section. The CGRP-IR nerve fibres were found mainly in dermis, too few being present in epidermis to allow a statistical evaluation. The total mean density of CGRP-IR nerve fibres was found to be 40 per section (Fig. 2).

**CFS group I.** The mean number of PGP9.5-IR nerve fibres was found to be increased compared with the control animals in both the epidermis (135,  $p \leq 0.001$ ) and the total skin (312,  $p < 0.001$ ) (Fig. 2). The mean number of CGRP-positive nerve fibres of the skin was also increased (66,  $p < 0.001$ ).

**CFS group II.** The mean number of PGP 9.5-IR nerve fibres was increased both in the epidermis (140,  $p \leq 0.001$ ) and in the total skin (291,  $p < 0.001$ ) (Fig. 2). The number of cutaneous CGRP-IR nerve fibres was also increased (66,  $p < 0.001$ ).

#### Immunocytochemistry of the dorsal root ganglia

As the sectioned area differed between the various DRG specimens (Fig. 3), the density of the IR cells was expressed as a ratio of positive versus negative cells.

There was no difference between the ganglia of the group of animals treated by low dose CFS (I) compared with the control animals (Fig. 4). The IR for PGP 9.5, CGRP and VR1 in the ganglia of animals in experimental group II is exemplified in Fig. 3. The results are summarized in Fig. 4, showing that the proportions of PGP 9.5-IR cells had increased as compared with the control ganglia (41 vs 35%;  $p=0.002$ ). The proportion of CGRP-IR cells had also increased (37 vs 33%;  $p=0.003$ ), as well as the number of VR1-IR cells (46 vs 42%;  $p=0.004$ ).

#### DISCUSSION

In the present study, both treatment regimens, involving either 0.13 mA or 0.53 mA, led to a proliferation of

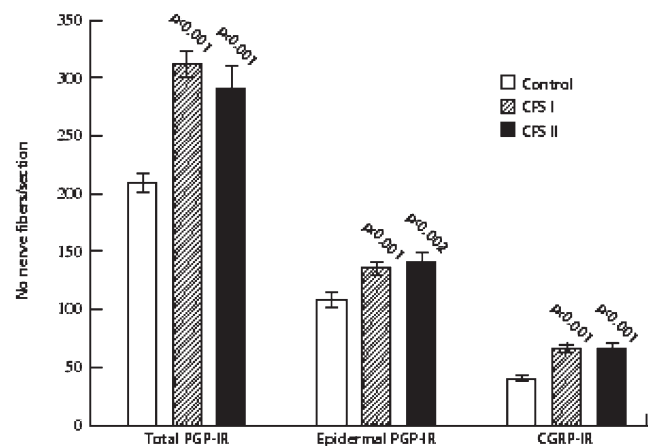


Fig. 2. Mean  $\pm$  SEM values for the pan-neuronal marker protein gene-product (PGP) 9.5-immunoreactive (IR) nerve fibers, the epidermal PGP 9.5-IR nerve fibers, and CGRP-IR nerve fibers in the skin of the back in the controls and in cutaneous field stimulation (CFS) groups I (0.13 mA) and II (0.53 mA).

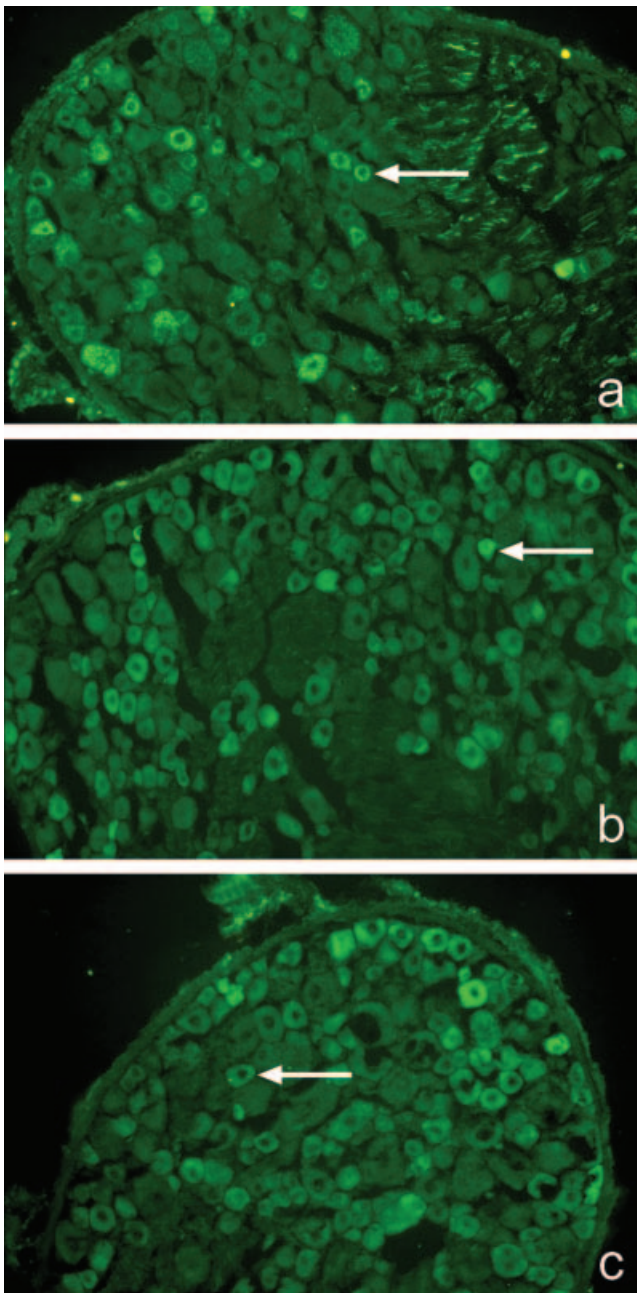


Fig. 3. Dorsal root ganglia (DRG) from experimental group II (0.53 mA) rats, showing PGP-IR (a), CGRP-IR (b), and VR1-IR (c). Examples of immunoreactive cells are shown by the arrows. Magnification  $\times 250$ .

sensory nerve fibres in both the epidermal and the dermal layer of skin of the rat. This contrasts with the results of a human study of the effects of CFS on itch where a reduction in epidermal PGP 9.5-IR nerve fibres was observed (10). Most patients receive CFS with a current of about 0.27–0.53 mA, the cathode covering only a small patch, less than palm size in area. In the present rat experiments, in contrast, the cathode covered the major part of the back. Additionally the current exerted its effects on a body weight that is only about 1/280 that of humans. It is

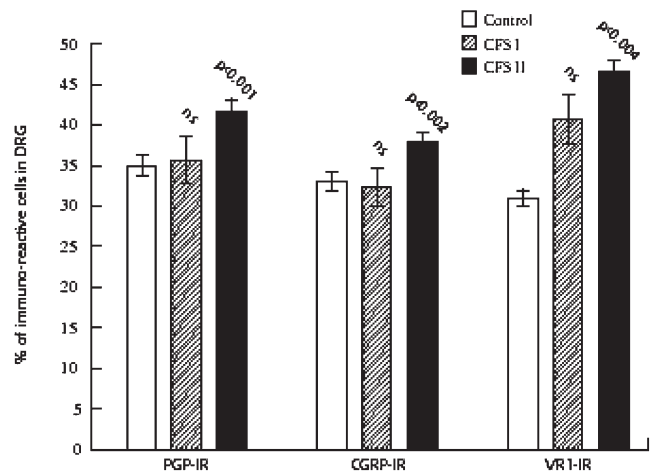


Fig. 4. Mean  $\pm$  SEM values for PGP-IR cells, CGRP-IR cells and VR1-IR cells in the ganglia of the controls and cutaneous field stimulation groups I (0.13 mA) and II (0.53 mA), respectively.

possible that such strong electrical stimulation of the skin of the rat is harmful, thus inducing a proliferation of nerve fibres in order to protect the skin. Proliferation of sensory nerve fibres has indeed been observed during the healing of tissue injury in the rat (11). Further, sprouting of epidermal nerve fibres has been reported in combination with localized pain and a burning sensation in patients with vulvar vestibulitis syndrome (14). However, although electric current can induce thermal damage, there were no signs of tissue injury in the present study.

In both of the treatment regimens, transmission of sensory information to the spinal cord might induce a segmental efferent response and thus conceivably give rise to antidromic C-fibre stimulation with ensuing local neurogenic inflammatory response (13). However, high-intensity CFS at 0.53 mA for 2 weeks was accompanied by stress-related behaviour and a lack of gain in weight in the rats, whereas animals treated with the lower amplitude current (0.13 mA) did not behave differently from the control group. Also, whereas high-intensity CFS at 0.53 mA for 2 weeks was accompanied by an up-regulation of sensory neuron markers (PGP 9.5-CGRP and VR1-IR) in the DRG, no such up-regulation could be observed in the group treated with the lower amplitude current. One explanation for these findings could be that electrical stimulation of higher intensity is required to up-regulate neuronal messengers and receptors in DRG neurons. Another possibility is that high-intensity electrical stimulation not only activates neurons that issue the C-fibres but also those that project larger diameter fibres, like A-fibres (15, 16). Even larger myelinated primary afferents in rats, such as A $\delta$ -fibres, have been shown to express the VR1 receptor (17).

A third possibility is that the stressful behaviour and deficient weight gain influences the limbic system,

including the thalamus. Such stimulation might activate DRG by way of descending pathways. Apart from the stress behaviour found in the experimental group treated with high amplitude CFS, we do not know how the treatment is perceived by the animals. In contrast to treatment by capsaicin, the animals did not display any scratching behaviour that creates wounds on the head or neck (18).

In conclusion, high-intensity cutaneous electrical stimulation in the rat causes proliferation of cutaneous sensory nerve fibers and up-regulates sensory neuron markers in the DRG. CFS stimulation of moderate intensity causes proliferation of cutaneous sensory nerve fibers in the rat but does not produce any overt changes in the neuronal marker expression of DRG. Serial treatment with CFS is thus not likely to activate DRG in humans.

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