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The time course of CO_2 laser-evoked responses and of skin nerve fibre markers after topical capsaicin in human volunteers

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ABSTRACT

Objective: To assess the temporal relationship between skin nerve denervation and regeneration (dermal and intra-epidermal fibres, IENF) and functional changes (CO_2 laser-evoked potentials, LEPs, and quantitative sensory tests, QST) after topical cutaneous application of capsaicin.
Methods: Capsaicin (0.075%) was applied to the lateral calf for three consecutive days. QST, LEPs and skin biopsies were performed at baseline and time intervals up to 54 days post-capsaicin treatment. Biopsies were immunostained with antibodies for PGP9.5, TRPV1, and GAP-43 (marker of regenerating nerve fibres), and analyzed for IENFs and dermal innervation (for GAP-43).
Results: At 1 day post-capsaicin, cutaneous thermal sensitivity was reduced, as were LEPs. PGP9.5, TRPV1, and GAP-43 immunoreactive-nerve fibres were almost completely absent. By Day 12, LEPs had fully recovered, but PGP9.5 and TRPV1 IENF continued to be significantly decreased 54 days post-capsaicin. In contrast, dermal GAP-43 immunoreactivity closely matched recovery of LEPs.
Conclusions: A good correlation was observed between LEPs and GAP-43 staining, in contrast to PGP9.5 and TRPV1. Laser stimulation is a non-invasive and sensitive method for assessing the initial IENF loss, and regenerating nerve fibres.
Significance: Assessing skin biopsies by PGP9.5 immunostaining alone may miss significant diagnostic and prognostic information regarding regenerating nerve fibres, if other approaches are neglected, e.g. LEPs or GAP-43 immunostaining.

1. Introduction

Small-fibre neuropathy (SFN) has been described as a subtype of sensory neuropathy with a number of known causes and needing special investigation, since it affects C and Aδ-fibres which cannot be assessed directly with conventional nerve conduction studies. However, a number of investigative tools have been developed over the two last decades to help with the diagnosis of small-fibre neuropathies (Lacomis, 2002). The purpose of the present study was to determine the validity and sensitivity of CO_2 laser stimulation for diagnostic and pharmacological investigations in a "volunteer model" of SFN, and its relationship to quantitative sensory tests and skin nerve fibre markers.

Brief (millisecond) laser stimuli selectively activate Aδ and C-nociceptors in the epidermal layer of skin (Bromm et al., 1984), and evoke time-locked brain responses, i.e. laser-evoked brain potentials (LEPs; Carmon et al., 1976; Plaghki and Mouraux, 2005). A validated experimental model of chemical axotomy produces reversible superficial skin denervation by topical capsaicin application in healthy subjects (Nolano et al., 1999; Polydefkis et al., 2004). Repeated topical application of capsaicin has been shown to elevate warm detection and heat pain thresholds (Carpenter and Lynn, 1981; Lynn, 1990; Simone and Ochoa, 1991),

Abbreviations: CDT, cool detection threshold; CPT, cold pain threshold; ER, endoplasmic reticulum; GAP-43, growth associated protein-43; HPT, heat pain threshold; IENF, intra-epidermal nerve fibre; IENFd, intra-epidermal nerve fibre linear density; IF, immunofluorescence; IHC, immunohistochemistry; LEP, laser-evoked potential; PGP9.5, protein gene product 9.5; QST, quantitative sensory testing; RT, reaction time; SEF, sub-epidermal nerve fibre; TRPV1, transient receptor potential vanilloid 1; WDT, warm detection threshold.

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and to cause reduction of LEPs (Beydoun et al., 1996), followed by their restoration when topical capsaicin is discontinued.

In this study, two particular aspects were investigated in the cycle of nerve degeneration followed by regeneration following application of topical capsaicin: (1) the temporal relationship between functional and morphological measures, and (2) the sensitivity of these measures to change during the regeneration of the epidermal fibres. The functional measures used were psychophysical (quantitative sensory testing, QST) and electrophysiological (LEPs). Morphological measures were based on the quantification of small nerve fibres in punch biopsies of skin. Intra-epidermal nerve fibre density (IENFd), assessed by PGP9.5 immunohistochemistry, has been validated as a reproducible marker of SFN with high sensitivity and specificity as compared to other diagnostic tools (Lauria et al., 2005; Vlcková-Moravcová et al., 2008; England et al., 2009). Following topical capsaicin application, there is a rapid and profound loss of epidermal nerves. After discontinuation, the regenerative process starts but, in contrast to the functional variables, recovers very slowly over weeks (Nolano et al., 1999) to months, and is usually still incomplete at 6 months (Polydefkis et al., 2004). Although the time lag between functional and morphological recovery after topical capsaicin has been reported by several authors (Simone et al., 1998; Nolano et al., 1999; Khalili et al., 2001; Malmberg et al., 2004), it has, to our knowledge, never been investigated using electrophysiological methods such as LEPs, or histochemically with the nerve markers TRPV1 (heat/capsaicin receptor) and GAP-43 (marker of regenerating nerve fibres).

2. Methods

2.1. Subjects

Twelve paid healthy volunteers, with a mean age of 42 years (age range 30–55; 7 men) were included in the study. Ten of these subjects were right-handed with a body mass index (mean ± SD) of 25.3 ± 4.1 kg/m^2. The subjects had no history of alcohol or drug abuse, significant illnesses, or clinical findings suggestive of peripheral or central nervous system disorders. All had normal routine lab testing (blood sampling and urine analysis). Written informed consent was obtained from all subjects. The Local Ethics Committee approved the study.

2.2. Experimental design

After having given informed consent, subjects underwent a screening evaluation to assess their eligibility for study participation. The screening evaluation included a medical history, concomitant medication recording, physical and neurological examination and routine laboratory testing. Within 14 days prior to the start of capsaicin application, baseline QST, CO_2 laser stimulation and skin punch biopsies were performed on the non-dominant leg (Fig. 1a). The contralateral leg served as a control.

All subjects had normal QST and LEPs at the screening evaluation and were invited to receive a topical capsaicin application on three consecutive 24-h cycles (total 72 h). At the end of the third cycle (Day 1), subjects visited the clinical unit for CO_2 laser stimulation, QSTs and skin biopsies (Fig. 1a).

In this study, neurodegeneration after chronic topical capsaicin application was defined by the "absence" of Aδ-fibre laser activation, i.e. (1) no pricking pain, (2) a detection rate of less than 5% with reaction times (RTs) shorter than 750 ms and (3) a reduction in amplitude of the late (Aδ-fibre related) and/or ultra-late (C-fibre related) N2–P2 complex in the laser-evoked vertex potentials by at least 95% (see below). All volunteers fulfilled these criteria.

Subjects were re-assessed at regular intervals thereafter, i.e. Day 5, Day 12, Day 26 and Day 54 for QST and CO_2 laser stimulation. Skin punch biopsies were performed on fixed time points

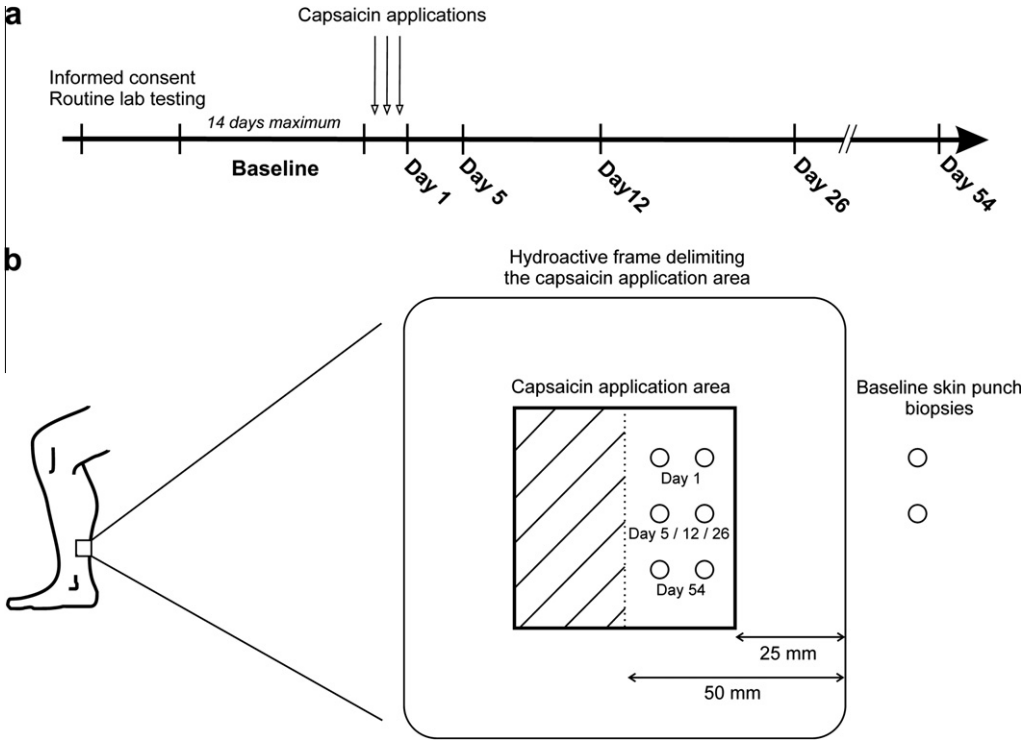

Fig. 1. (a) Experimental schedule. When the volunteers met the inclusion criteria, they received topical capsaicin applied to the distal calf during three consecutive 24-h cycles. Subjects were subsequently assessed at regular intervals with QST, laser stimulation and skin punch biopsies. (b) Capsaicin model and testing sites. An occlusive bandage (10 × 10 cm) was applied to the lateral distal calf, approximately 10 cm above the lateral malleolus of the non-dominant leg. Thermal threshold testing and CO_2 laser stimulation were performed in the dashed part of the capsaicin application area. Skin punch biopsies were performed in the other half of the capsaicin application area.

Day 1 and Day 54 and at one additional time point in between, on Day 5 or Day 12 or Day 26.

2.3. Topical capsaicin application procedure

An occlusive hydroactive bandage (DuoDerm 10 × 10, ConvaTec, Deeside, UK) measuring 10 × 10 cm with a central window of 50 × 50 mm was applied to the lateral site of the distal calf, approximately 10 cm above the lateral malleolus of the non-dominant leg of each subject (Fig. 1b). The fenestrated area of this montage was filled with approximately 3.5 g of capsaicin cream (Axsain 0.075%, Cephalon Ltd., Oxford, UK) and covered for 24 h with a transparent dressing (Tegaderm 6 × 6 cm, 3 M Health Care, Neuss, Germany). After that period, the transparent dressing and remaining cream were removed and replaced. This procedure was repeated twice to obtain a total of 72 consecutive hours of capsaicin cream exposure. The procedure was well tolerated in all volunteers but one, who presented with a slight allergic eczema during the study. A causative role of the capsaicin cream formulation or the occlusive bandage itself could not be excluded.

2.4. Skin punch biopsies

Nine skin punch biopsies (diameter 4 mm) were performed on each subject under local anesthesia (Lidocaine 1%) and aseptic techniques by an experienced investigator in the clinical unit. Prior to capsaicin application (baseline), two biopsies were taken from the non-dominant calf and one from the contralateral leg as a control. After capsaicin application, only the non-dominant calf was considered and biopsies were performed, as shown in Fig. 1b, on one half of the capsaicin application area. Two pairs of biopsies were taken at fixed time points, i.e. Day 1 and Day 54, and one pair at a randomly chosen time point either Day 5 (1 subject) or Day 12 (6 subjects) or Day 26 (5 subjects). According to the methods used by Polydefkis et al. (2004), a distance of $\geqslant$0.5 cm between the biopsies and from the borders of the skin region exposed to capsaicin was respected in order to reduce the "contamination" of collateral reinnervation by sprouting.

Two different processing techniques were used for subsequent immunohistochemical purposes. One biopsy per time point was Zamboni-fixed and further processed as described below. The other biopsy was snap frozen in optimum cutting tissue (OCT) compound (Sakura TissueTek Europe, Zoeterwoude, the Netherlands) and used for the TRPV1-staining procedures. Both processing techniques were always used, except for the single sample on Day 5, in which, for technical reasons, only PGP9.5 staining was performed.

2.5. Immunohistochemistry/immunofluorescence

2.5.1. PGP9.5 immunohistochemistry/immunofluorescence

For immunohistochemistry (IHC) at HistoGeneX, 16 μm serial sections were air dried and placed in the autostainer (DAKO, Denmark). After endogenous peroxidase blocking, sections were incubated with anti-human PGP9.5 (Table 1) and visualized by a B-labeled secondary system and ABC detection (Vectastain ABC kit, Vector Laboratories, Ltd., UK), using DAB (DAKO, Denmark) as a chromogen. Tissue sections were counterstained using neutral red (Sigma–Aldrich, Inc.). For immunofluorescence (IF), tissue sections were incubated with the same primary antibody and visualized by a Cy_3-labeled secondary antibody (Jackson Immunoresearch Laboratories, Inc., PA, USA). Counterstain was performed using Hoechst (Invitrogen Corporate, Molecular Probes, California, USA). Quality control steps were included for all stains to validate each run.

Table 1
List of primary antibodies and dilutions used in this study.

Antibody	Host	Source	Titre
HistoGeneX, Antwerp, B			
PGP9.5	Rabbit	Ultraclone: RA 95/101	1:5000
Hammersmith Hospital, London, UK			
TRPV1	Rabbit	GSK: C22	1:10,000
GAP-43	Mouse	Sigma G 9264: clone 7B10	1:100,000

Image analysis was performed using Mirax Virtual Slide scanner (for IHC, minimally 5 × 2500 μm epidermis length) (Carl Zeiss, Germany) and the Axiovision Mozaik Imaging Software (Axiovision Rel 4.6) (for IF, minimally 3 × 2500 μm epidermis length) (Carl Zeiss, Germany). The quantification method used in this study was modified from the EFNS guidelines (Lauria et al., 2005) since thinner tissue sections were handled. Intra-epidermal nerve fibres (IENF) included all single stained nerve fibres crossing the dermal–epidermal junction (defined here as transbasal membrane nerve fibres, TBMNF). In addition, clearly branched epidermal nerve fragments that did not cross the basement membrane were also counted. This method was used throughout the entire study, from baseline to Day 54. Tissue sections of skin biopsies in our studies are processed at 15–16 μm thickness (exception made for the GAP-43 staining, see below) to enable staining for multiple markers in the same biopsy, with a number of sections for each marker (Facer et al., 1998, 2007; Atherton et al., 2007).

2.5.2. TRPV1 and GAP-43 immunohistochemistry

At Hammersmith, tissues were supported in OCT medium (RA-Lamb Ltd., Eastbourne, UK) to allow for best orientation. Frozen sections (15 μm for TRPV1 post-fixed in freshly prepared, 4% w/v paraformaldehyde in 0.15 M phosphate-buffered saline (PBS) for 30 min and 30 μm for GAP-43 pre-fixed in Zamboni) were collected onto poly-L-lysine (Sigma, Poole, UK) coated glass slides. Thicker sections were used for GAP-43 in order to increase the sensitivity of this marker in the epidermis. Endogenous peroxidase was blocked by incubation in Industrial methylated spirit containing 0.3% w/v hydrogen peroxide for 30 min (for both frozen and Zamboni-fixed specimens). After rehydration, sections were incubated overnight with primary antibodies (Table 1). Sites of primary antibody attachment were revealed using nickel-enhanced, avidin–biotin peroxidase (ABC – Vector Laboratories, Peterborough, UK) as previously described (Facer et al., 2007). For specificity studies (TRPV1), experiments were performed with the primary antibody incubated with antigenic peptide for 2 h prior to incubation with the sections. Omission of primary antibodies and sequential dilution of antibodies gave appropriate results, as in previous publications (Smith et al., 2002; Facer et al., 2007). The monoclonal GAP-43 antibody recognises an epitope present on both kinase C phosphorylated and dephosphorylated forms of GAP-43 (Sigma product G9264 datasheet, clone 7B10); it immunostains and reacts with GAP-43 in immunoblots, and detects increase of GAP-43 in a cell injury model (Meiri et al., 1991; Galbiati et al., 1998). Sections were counterstained for nuclei in 0.1% w/v aqueous neutral red and mounted in xylene-based mountant (DPX; BDH/Merck, Poole, UK), prior to analysis.

TRPV1 intra-epidermal fibres were counted as described above for PGP9.5 and expressed as fibres per mm length of epidermis. For sub-epidermal GAP-43 fibre analysis digital photomicrographs were obtained using an Olympus BX50 microscope. Olympus analySIS® FIVE software was used to define and measure a sub-epidermal area with a depth of 200 μm below the basal epidermis. This software was also used to measure length of each of the fibres within the defined area. GAP-43 immunoreactive fibre numbers

were counted manually within the same area. Values were expressed as total number and total length of fibres per square millimetre.

2.6. Quantitative sensory testing

QST was limited to the assessment of thermal perception using a contact thermode measuring 30 × 30 mm (TSA 2001-II, Medoc, Israel). Stimuli were delivered at the distal calf as shown in Fig. 1b on one half of the capsaicin application area in the treated leg, and on a mirror site in the contralateral leg. A baseline thermode temperature of 32 °C and a heating rate of 1 °C/s were used. Thresholds were measured according to the method of limits previously described by Fruhstorfer et al. (1976). Briefly, increasing or decreasing heating ramps were applied to the skin. For each stimulus the subject was instructed to press a button that reversed the thermal stimulation as soon as he/she detected a change in skin temperature, either cool detection threshold (CDT) or warm detection threshold (WDT), or as soon as the stimulation became painful (cold pain threshold or CPT and heat pain threshold HPT). CDT and WDT were estimated by the mean of four successive stimulus presentations. CPT and HPT were estimated by the mean of three successive stimulus presentations. An inter-stimulus interval of 6–8 s was used when testing perception thresholds whereas intervals of 15–20 s were used for HPT and of 20–30 s for CPT. To prevent tissue damage, the maximum and minimum temperatures were set at 50 °C and 5 °C.

2.7. CO_2 laser stimulation

CO_2 laser stimuli were applied to the same sites used for QST as shown in Fig. 1b. The CO_2 laser was designed and built in the Department of Physics of the Université catholique de Louvain (for a short description, see Plaghki et al., 1994).

In a first series of laser stimuli, the absolute detection threshold, defined as the lowest stimulus intensity detected with a probability of 0.5, was determined using the method of limits. Then, a series of 30 stimuli (surface area: 79 mm^2; duration: 50 ms; intensity of 10 ± 1 mJ/mm^2) producing a clear pricking sensation ("first pain" which is Aδ-nociceptor related) in normal subjects were applied on each site (Fig. 1b). Inter-stimulus interval was 6–12 s. During that interval, the laser beam was slightly displaced randomly to avoid sensitization and habituation. Skin laser stimulation was well tolerated, though a slight hyperemic skin reaction was sometimes observed in some volunteers after laser stimulation, which resolved within a few hours. Reaction times (RTs) were measured by instructing the subject to press a micro-switch mounted on a hand-controller as soon as he/she perceived any type of sensation at the stimulation site.

2.8. Laser-evoked potentials

Laser-evoked brain potentials were recorded from 19 Ag–AgCl scalp electrodes, based on the international 10–20 system, with linked earlobes (A1/A2) as reference. Impedance was kept below 5 kΩ. In addition, electro-oculogram (EOG) of the right eye was recorded with disposable Ag–AgCl surface electrodes (Nessler Medizintechnik, Innsbruck, Austria). Evoked potentials, EOG, RTs and laser trigger signals were recorded on a PLEEG (Walter Graphtec, Germany). Signals were filtered (low-pass 75 Hz) and digitized at 167 counts per second with a 10-bit resolution. Data were stored on disk and analyzed off-line with the BrainVision Analyzer (Brain Products GmbH, Germany). The time window for analysis was –500–2500 ms according to laser stimulus onset. All trials containing EOG artefacts were rejected from subsequent analysis after visual inspection. Artefact-free trials were time averaged and corrected for baseline offset using the pre-stimulus interval of 500 ms for each channel.

For every subject, latency (in ms) and amplitude (in μV) of evoked potentials was measured on the averaged waveform for each site. Latencies were measured from stimulus onset to peak. Amplitudes were measured from peak to the averaged amplitude of the 500-ms pre-stimulus interval. Three well-known LEP components were individualized: N1, N2 and P2. Component P2 was identified at electrode C_Z as the positive component with maximal amplitude between 300 and 500 ms after stimulus onset. N2 was then defined at electrode C_Z as the negative component preceding P2 and occurring between 150 and 300 ms after stimulus onset. N1 was then defined at the temporal electrode contralateral to the stimulation site as well as by trace superposition, as the negative deflection preceding N2 between 120 and 200 ms after stimulus onset.

2.9. Statistical analysis

Repeated measures analysis of variance (ANOVA) was used for estimating differences in morphological, psychophysical and electrophysiological data at the different time points. Fisher's least significant difference (LSD) procedure was used for comparing treatment group means when the ANOVA null hypothesis for equal means was rejected. Bivariate correlations were assessed using Pearson's correlation coefficient. When testing for equality of means of IENFd, CDT/WDT or LEPs between different skin areas, paired *t*-tests or non-parametric Wilcoxon signed-rank tests were used (α-level = 0.05). Statistical analyses were performed with SigmaStat software version 3.5 (Systat Software, San Jose, CA).

3. Results

3.1. Nerve fibre densities in skin biopsies

At baseline, skin biopsies taken from the capsaicin-treated leg of the 12 volunteers yielded a significantly higher IENFd using immunofluorescence of PGP9.5 (Mean IF IENF/mm ± SD: 13.8 ± 7.02) as compared to the chromogen assay (Mean IHC IENF/mm ± SD: 6.6 ± 5.04) ($z = -0.483$; $p < 0.013$). Furthermore, there was no significant difference observed in baseline IENF/mm between the dominant (control) and non-dominant leg for both staining techniques ($z = -0.454$; $p = 0.65$). All subsequent data and analyses of PGP9.5 IENFd will be restricted to the IF assay. The number of fibres (mean ± SD) immunoreactive to TRPV1 in the epidermis and GAP-43 in the sub-epidermis was 7.4 ± 3.8/mm and 32.9 ± 8.52/mm^2, respectively.

After 72 h of topical capsaicin application (Day 1), IENFd, based on PGP9.5 staining, dramatically decreased towards 7 ± 4% of baseline (Fig. 2). Twelve days later a very slight but non-significant increase was observed. During the two following weeks there was a considerable and significant change in the rate of regeneration as at Day 26 the density of IENFd reached 40 ± 20% of baseline. Subsequently, the regeneration rate waned as by Day 54 IENFd density reached on average 56 ± 39% of baseline. A one-way repeated measures ANOVA indicated that IENFd had significantly increased at Day 54 compared with Day 1 but not at Day 12 and Day 26. The early stage of regeneration was characterized by an increase in transbasal membrane nerve fibres followed at a later stage by branching of those fibres and a concomitant increase in IENFd.

TRPV1 immunoreactive IENFd showed a similar pattern of decrease and recovery as PGP9.5 stained fibres (Figs. 2 and 3). Following application of capsaicin (Day 1) there was a dramatic reduction in TRPV1 immunoreactive IENFd (mean ± SD 2.7 ± 1.5% of baseline

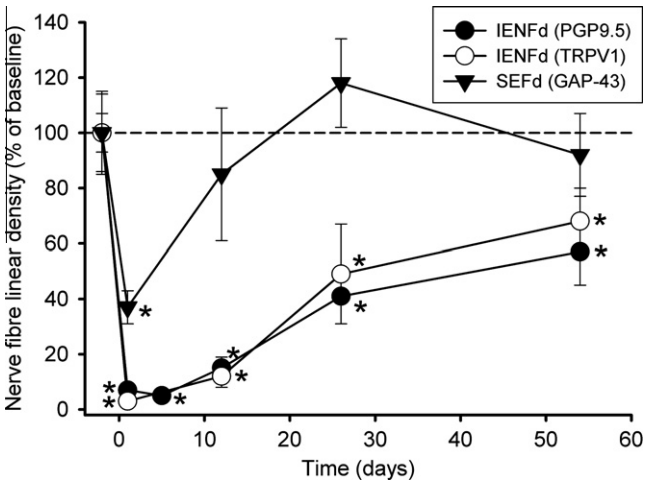

Fig. 2. Plot of nerve fibre densities (% of baseline values, mean ± SE) as a function of time after three consecutive days of topical capsaicin using different staining techniques (PGP9.5 and TRPV1 IENF and GAP-43 SEF). The number *n* of samples per time point was as follows: $n = 12$ for baseline, Day 1 and Day 54, $n = 6$ for Day 12, $n = 5$ for Day 26 and $n = 1$ for Day 5 (for technical reasons, TRPV1 and GAP-43 staining was not performed on Day 5).

values) followed by a progressive increase reaching a mean ± SD of 68.2 ± 42% on Day 54.

GAP-43 antibodies did not stain intra-epidermal nerve fibres, though a few fibres appeared to enter the basal keratinocyte layer. The mean (±SD) number and length of GAP-43 immunoreactive sub-epidermal fibres decreased on Day 1 to 36.5 ± 6.2% and 38.8 ± 8% of baseline values, respectively. There was a rapid recovery of GAP-43 immunoreactive fibres (number of fibres/mm^2: mean ± SD % of baseline values: 85 ± 24.2 on Day 12, 118 ± 15.5 on Day 26 and 92 ± 14.5 on Day 54; total length of fibres/mm^2: mean ± SD % of baseline values 96 ± 35.1 on Day 12, 120 ± 41.5 on Day 26 and 99 ± 18.5 on Day 54) (Figs. 2 and 3).

The morphological appearance of intra-epidermal and sub-epidermal nerve fibres was fragmented and very few axonal swellings were observed in the early stages. Using PGP9.5 immunostaining, up to three sub-epithelial fibres per biopsy showed axonal swellings in 3/11, 2/5 and 1/4 biopsies at Day 1, 12 and 26, respectively. Similar swellings were seen in only one intra-epithelial fibre and in only one biopsy, at Day 12. Axonal swellings were not detected in any biopsy at Day 54.

Skin biopsies were also screened for inflammation. No pathological inflammatory reaction was observed in all except one subject who showed a mild perivascular and perifollicular inflammatory reaction without epidermal involvement, as a consequence of slight allergic eczema during the study.

3.2. Quantitative sensory testing

Results from QST are shown in Table 2. On the non-dominant leg, CDT at baseline was on average 30.0 ± 0.98 °C, which means that, starting at 32 °C, a decrease in skin temperature of less than 1 °C was readily detected. WDT was 37.7 ± 2.79 °C and HPT was 47.5 ± 1.26 °C. However, this threshold assessment was possibly flawed by the fact that, for safety reasons, there was an upper limit of 50 °C imposed on the heating ramp of the thermode. For the same reason, the lower limit of the cooling ramp was set at 5 °C. Ten subjects did reach that low temperature without reporting pain. That is why we had to dismiss the assessment of cold pain thresholds.

These QST assessments at baseline for the control leg yielded similar threshold values. At Day 54, WDT was slightly increased ($t = -1.118$; $p = 0.29$) whereas CDT remained stable ($t = 0.802$; $p = 0.43$).

The 72-h consecutive capsaicin application had a significant influence on cool and warm thresholds as shown in Table 2. At Day 1, CDT increased significantly by an average of −2.4 °C cooler compared to baseline values. There was no significant difference between Day 5 and baseline CDT ($p = 0.198$). However, at all later time points, CDT remained significantly increased (lower temperature) as compared to baseline ($p = 0.004$ at Day 12, $p = 0.013$ at Day 26 and $p = 0.011$ at Day 54).

WDT evolved in a similar fashion as CDT after capsaicin application. It increased significantly at Day 1 (+4.7 ± 3.66 °C) and evolved progressively towards baseline values by the following time points. WDT remained significantly different from baseline ($p = 0.008$ at Day 12, $p < 0.001$ at Day 26) until Day 54 ($p = 0.074$).

The imposed upper limit of 50 °C on the heat ramp interfered with the determination of the HPT after capsaicin application. Indeed, five subjects reached this limit without reporting pain at Day 1, two subjects at Day 12 and one subject at Day 26. Given the missing data points for these subjects, HPT data were not taken into consideration.

3.3. Detection thresholds, detection rates and reaction times for laser stimuli

The average *absolute detection threshold* (C-fibre related) for CO_2 laser stimulations at baseline was of 4.5 ± 0.6 for the capsaicin-treated leg. The temporal evolution after topical capsaicin applications for the absolute detection threshold is summarized in Table 3 (first row). One-way repeated measures ANOVA revealed a highly significant ($F = 35.382$; $p < 0.001$) increase of absolute detection thresholds at Day 1 compared to baseline and a fast return to lower values as early as Day 5 ($p = 0.643$). The thresholds returned to baseline values from Day 12 onwards.

The average *pricking pain threshold* (Aδ-fibre related) was 7.5 ± 1.1 mJ/mm^2 for the leg. The temporal evolution after topical capsaicin applications for the pricking pain threshold is also summarized in Table 3 (second row). At Day 1, neither painful nor pricking sensations were felt by the volunteers therefore no Aδ-fibre-related activation thresholds could be determined, as for safety reasons no laser stimuli above 12 mJ/mm^2 were delivered. The threshold for pricking pain returned to baseline values from Day 5 onwards.

At baseline the total detection rate for laser stimuli was 90% and 92%, for the dominant and non-dominant leg, respectively. The total detection rates after exposure to capsaicin are reported in Table 3 (row 3). At Day 1, there was an important reduction in detection performance to 23%. The detection performance increased from Day 5 on, but was still below baseline values at Day 54. The trade-off between RTs ascribed to Aδ-nociceptor activations and those in response to C-nociceptor activations was set at 750 ms according to previous studies (Mouraux and Plaghki, 2004; see also Fig. 4). The detection rates with RTs < 750 ms, and thus Aδ-fibre related, are also reported in Table 3 (row 4). These results are better illustrated by the relative frequency distributions of RTs as shown in Fig. 4. As commonly observed, these frequency distributions are fairly skewed with a long tail to the right. As already shown in Table 3, most RTs (⩾80%) were shorter than 750 ms and could only result from the activation of Aδ-nociceptors. The ±10% of RTs with longer latency were possibly misses or either resulted from the selective activation of the much slower conducting C-nociceptors. At baseline, the peak latencies of RTs reached 370 ms for the control leg and 378 ms for the contralateral leg. At Day 1, as shown in Table 3 (row 4) and Fig. 4 (first column), still 23% of the 360 stimulations were detected by the subjects, but less than 3% (2.8%) had a RT below 750 ms. The remaining RTs were

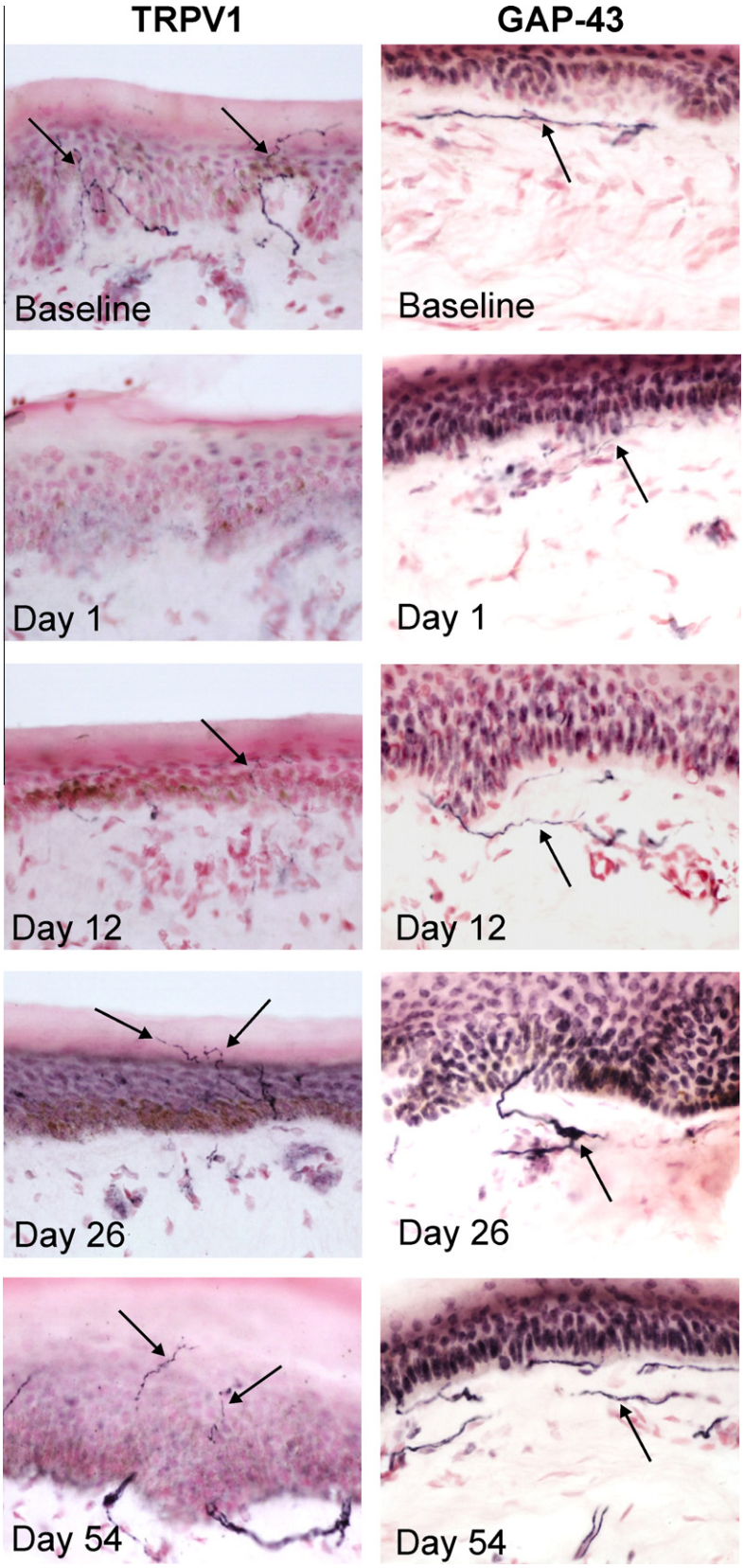

Fig. 3. TRPV1 IENF nerve fibres and sub-epidermal GAP-43 nerve fibres (arrowed) in calf skin before (Day 0) and after capsaicin application (Days 1, 12, 26 and 54). Magnification 40×.

considerably increased (peak value = 1403 ms) and spread over large time window. At Day 5, a more symmetrical bimodal distribution of RTs appeared with an overall stimulus detection rate of 67% (31.1% Aδ-fibre-related RTs peak value 465 ms). From Day 12

Table 2
Quantitative sensory testing results.

Thresholds	°C ± SD	Mean difference with baseline (°C)					Critical Δ[a] (°C)
	Baseline	Day 1	Day 5	Day 12	Day 26	Day 54	
Warm detection	37.7 ± 2.79	+4.7**	+3.6**	+3.1**	+4.3**	+2.1	*2.32*
Cool detection	30.0 ± 0.98	−2.4**	−0.5	−1.3**	−1.1*	−1.1*	*0.85*

[a] Fisher's PLSD.
* $p < 0.05$.
** $p < 0.01$.

Table 3
Detection thresholds (mJ/mm^2 ± SD), detection rates (%) and reaction times (ms) for CO_2 laser stimulation.

	Leg (capsaicin-treated)					
	Baseline	Day 1	Day 5	Day 12	Day 26	Day 54
C-related detection threshold (mJ/mm^2)	4.5 ± 0.6	8.3 ± 1.1	5.2 ± 1.1	4.0 ± 1.2	4.1 ± 0.4	4.1 ± 0.4
Aδ-related detection threshold (mJ/mm^2)	7.5 ± 1.1	[a]	8.0 ± 1.0	7.1 ± 1.0	7.4 ± 1.0	7.7 ± 1.0
Total detection rate (%)	92	23	67	78	79	85
Detection rate <750 ms (%)	82	<3	31	60	68	69
Reaction times (ms)	378	1403	465	394	359	352

[a] No Aδ-related detection threshold could be obtained for energy densities up to 12 mJ/mm^2.

on, the shape of the distributions appeared quite similar to the baseline with a return to normal peak values. At Day 54, overall detection rate at the capsaicin-exposed leg equaled 85% (peak value 352 ms) whereas the opposite control leg had a detection rate of 92% (peak value 342 ms).

3.4. Laser-evoked potentials

Baseline values of LEPs of both legs are reported in Table 4 and are within the range of those reported in the literature for similar stimulations and recording settings (Truini et al., 2005). There was no statistical difference in these values between LEPs obtained after stimulation of dominant and non-dominant legs. As all subsequent analyses are based on within subject comparisons we did not adjust LEP data for age and for height. Fig. 4 (middle and right column) shows, for one typical subject, the temporal evolution of LEPs recorded at C_Z and samples of his skin biopsies immunostained with PGP9.5.

Latencies of LEP components, when detectable, did not differ among time points ($p = 0.479$ for N1; $p = 0.149$ for N2 and $p = 0.360$ for P2).

N2–P2 amplitudes of LEPs varied dramatically after exposure to capsaicin (Fig. 5). At Day 1, there were no identifiable LEP complexes in the time-averaged records of 11 subjects. Only one subject presented a small late N2–P2 complex. Presence of late-LEPs was defined by the existence of a negative–positive biphasic signal within a characteristic time window (180–500 ms) and displaying a typical topographic scalp projection in the time-averaged EEG recordings time-locked to the laser stimulus. Of note there were no ultra-late-LEPs, i.e. N2–P2 complexes related to the selective and isolated activation of C-nociceptors. Already by Day 5, a late N2–P2 complex was identified in all but three subjects; the N2–P2 complex was 56% of baseline. From Day 12 on, the N2–P2 complex returned to baseline values (Fig. 5).

3.5. Correlations between functional and morphological variables

The relationships between warm (WDT) and cool (CDT) detection thresholds vs IENFd using different staining methods are reported in Table 5. Most correlations were statistically significant. Nevertheless, the strength of the linear dependence is rather low. As expected, the correlations were negative for WDT (the lower IENFd the higher the temperature at WDT) and positive for CDT (the lower the IENFd the lower the temperature at CDT).

No significant correlations were found between absolute detection thresholds and Aδ-related pricking pain thresholds obtained after laser stimulation and skin nerve fibre densities, exception made for absolute detection threshold and GAP-43-stained SEF ($r = 0.434$; $p = 0.006$).

The N2–P2 amplitude of LEPs was correlated with skin nerve fibre densities measured using the three different markers. Results are reported in Table 5. There was a significant correlation between N2–P2 amplitude and IENFd based on PGP9.5, TRPV1 and SEF number of GAP-43 immunoreactive fibres.

Phase plots of relative (% of baseline values) N2–P2 amplitudes vs relative IENF densities are shown in Fig. 6. These diagrams illustrate the time lag between LEP amplitudes and IENF densities. During the process of nerve fibre regeneration, LEP amplitudes were clearly in advance of PGP9.5 (Fig. 6a) and TRPV1 (Fig. 6b) IENF densities. This was clearly in contrast with GAP-43 SEFd. Indeed, except for Day 1, N2–P2 amplitudes closely followed the changes in GAP-43 SEFd (Fig. 6c).

4. Discussion

The present study aimed at determining (1) the temporal relationship between functional tests based on CO_2 laser technology and morphological measures based on IENFd in skin biopsies and (2) the sensitivity to change of these tests using an experimental model of topical application of capsaicin. The results show that most functional and morphological measures were sensitive to the degenerative and subsequent regenerative processes, and confirm that morphological recovery with conventional staining lags well behind functional recovery. Most interestingly, the late-LEPs, in normal conditions exclusively related to the activation of Aδ-nociceptors, were exquisitely sensitive in the early regenerative stage (Figs. 4 and 5). Within 2 weeks of capsaicin discontinuation a full recovery of LEPs was observed compared to baseline. In contrast the regenerative process of small fibres in the skin was slower, and only half the number of baseline immunoreactive-nerve fibres for the conventional nerve marker PGP9.5 and TRPV1 were observed 5 weeks after capsaicin discontinuation. This early functional recovery (LEPs) was however reflected in the density of the sub-epidermal nerve fibres immunoreactive to GAP-43, indi-

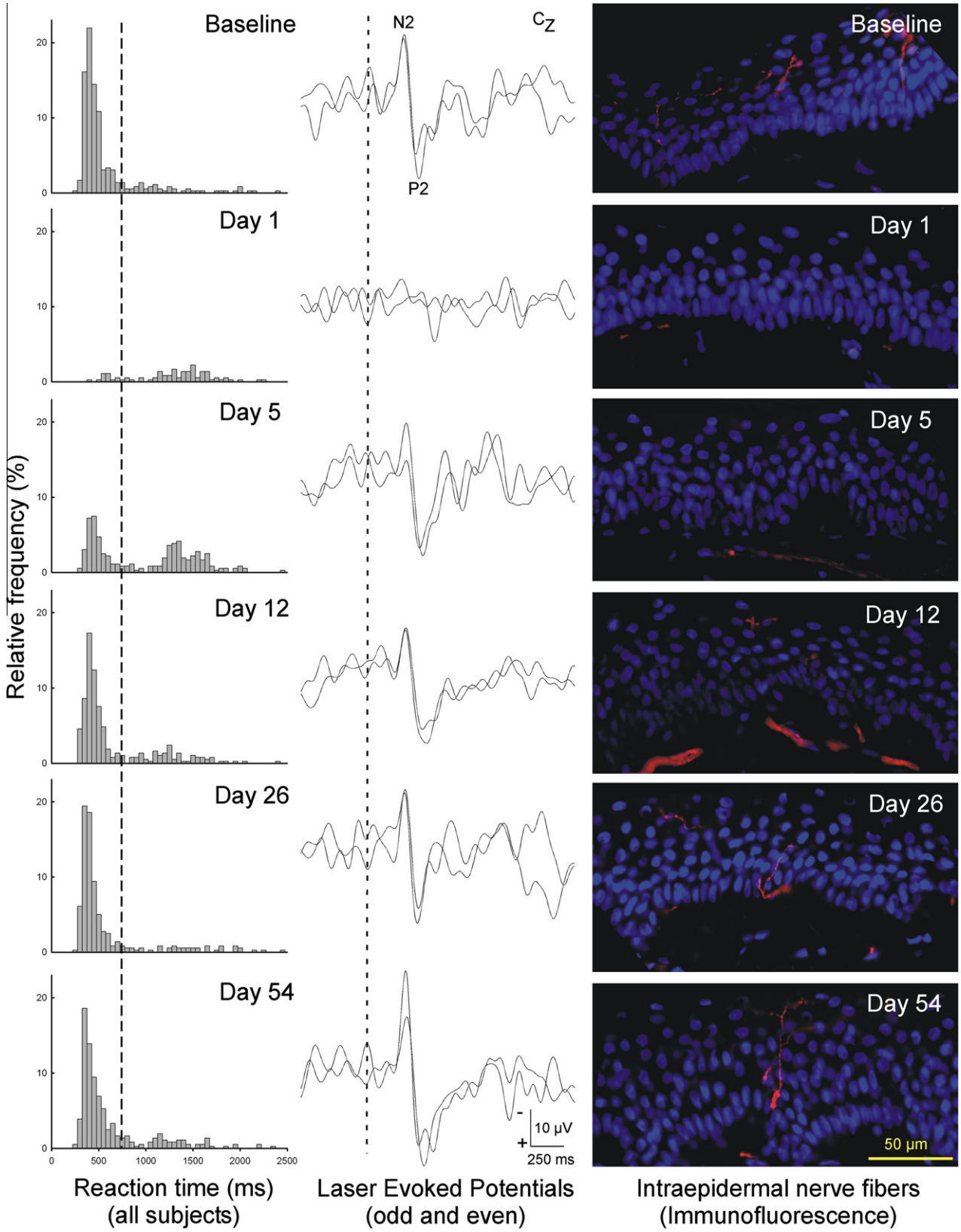

Fig. 4. (a) Relative frequency distributions of reaction times to laser stimuli supraliminal for Aδ-fibre activation at baseline and at different time points after three consecutive days of topical capsaicin. The vertical dotted line at 750 ms separates the domain of reaction times considered as responses to Aδ- or C-fibre activations, respectively. (b) Time-averaged LEPs (odd and even) recorded at the vertex (C_z–$A_{1,2}$) in one subject. After three consecutive days of topical capsaicin (Day 1), the N2–P2 complex was absent as compared to baseline. It clearly reappeared at Day 5 and recovered completely from Day 12 onwards. (c) Correspondent images of skin biopsies at different time points using immunofluorescence technique (PGP9.5 stained fibres in red, nuclear material counterstained in blue). Topical capsaicin resulted in a complete disappearance of intra-epidermal PGP9.5 fibres at Day 1 and Day 5. Partial reinnervation became evident only by Day 12.

Table 4
LEP components: latencies (ms ± SD) and amplitudes (μV ± SD) at baseline.

	Control leg	Capsaicin-treated leg
Latencies (ms)		
N1	203 ± 53	210 ± 32
N2	269 ± 34	273 ± 33
P2	445 ± 78	432 ± 71
Amplitudes (μV)		
N2–P2	22.1 ± 10.5	26.0 ± 11.4

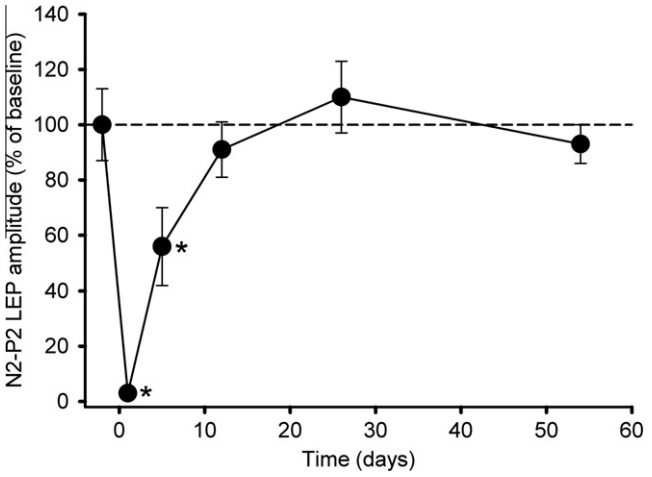

Fig. 5. Plots of N2–P2 amplitude of LEPs (% of baseline values, mean ± SE) as a function of time after three consecutive days of topical capsaicin.

Table 5
Correlative analyses between functional variables and skin nerve fibre densities.

Correlation variables	*r* value	*p* value
QST		
PGP9.5 IENFd vs CDT (°C)	0.438	0.002
PGP9.5 IENFd WDT (°C)	−0.359	0.013
TRPV1 IENFd vs CDT (°C)	0.514	<0.001
TRPV1 IENFd vs WDT (°C)	−0.523	<0.001
GAP-43 SEFd vs CDT (°C)	0.302	0.046
GAP-43 SEFd vs WDT (°C)	−0.413	0.005
LEP N2–P2 amplitude		
PGP9.5 IENFd vs LEP N2–P2 amplitude (μV)	0.417	0.004
TRPV1 IENFd vs LEP N2–P2 amplitude (μV)	0.539	<0.001
GAP-43 SEFd vs LEP N2–P2 amplitude (μV)	0.599	<0.001

cating high levels of neuronal growth cone-related proteins during axonal regeneration (Benowitz and Routtenberg, 1997).

The points of interest for discussion include: (a) the validity of topical capsaicin for 'modelling SFN' and (b) the mismatch between functional and morphological recovery.

4.1. Topical capsaicin as a model for studying SFN

The topical capsaicin model for SFN used in the present study followed the work of Polydefkis et al. (2004), who developed a standardized cutaneous nerve regeneration model. This allowed tests of pharmacological agents in short time periods, and small groups of healthy volunteers rather than large groups of neuropathic patients, with decreased regenerative rates of epidermal nerve fibres. However, our procedure differed as we applied 0.075% capsaicin cream on three consecutive days, instead of 0.1% capsaicin on 2 days, in order to meet our criteria of complete functional neurodegeneration.

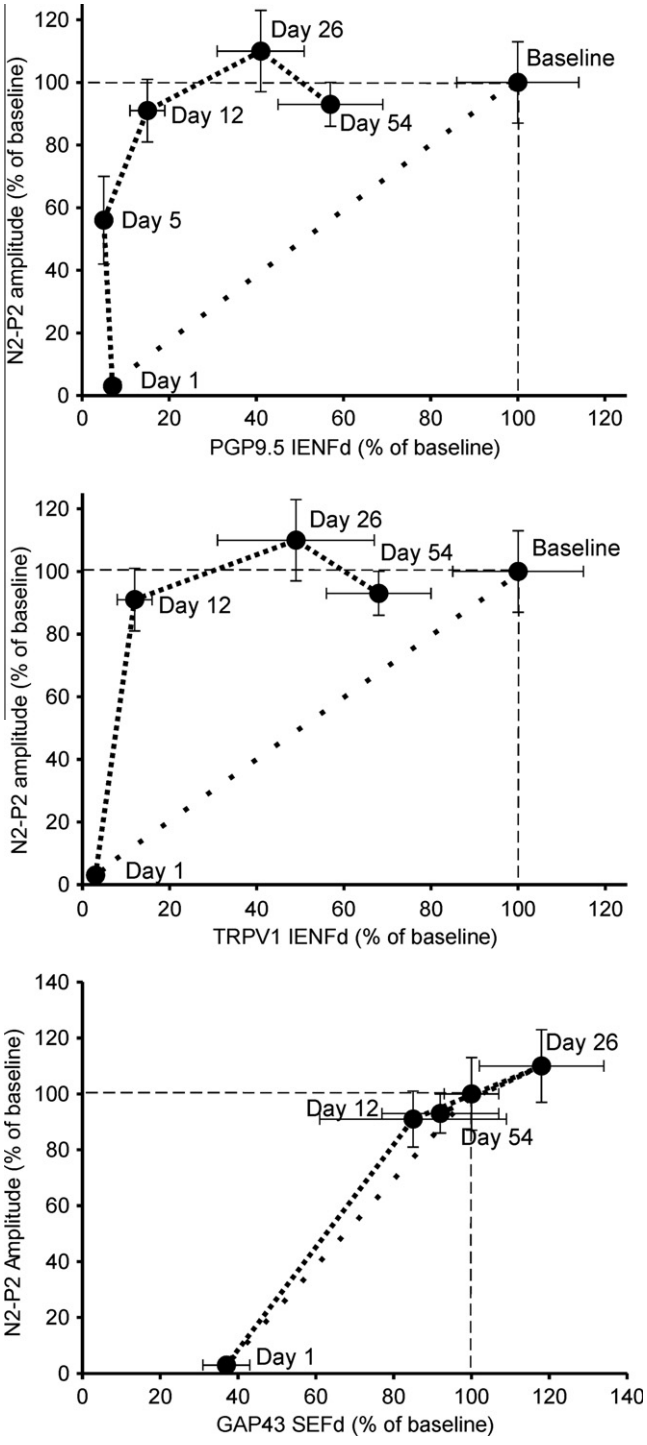

Fig. 6. Phase plots of N2–P2 amplitude of LEPs as a function of nerve fibre densities using three different staining markers: PGP9.5 IENF, TRPV1 IENF, and GAP-43 SEF, expressed as % of baseline values (mean ± SE) at different time points following topical capsaicin exposure. For technical reasons, TRPV1 and GAP-43 staining was not performed on Day 5. There was clearly a time lag between LEP amplitude and IENF density for PGP9.5 and TRPV1 staining. During the process of nerve fibre regeneration, the recovery of LEP amplitudes preceded the recovery of nerve fibres. However, this time lag was not seen in the lower plot where GAP-43 SEF density progresses in a similar fashion to LEP amplitudes.

The rationale for our model is based on work by Reilly et al. (1997), Simone et al. (1998) and Nolano et al. (1999) who demonstrated that the functional effects of capsaicin (i.e., burning pain and hyperalgesia followed by hypoalgesia with increased detection threshold and diminished heat pain sensation) were due to

destruction of epidermal nerve fibres in blister roofs (Reilly et al., 1997) and skin biopsies, either after injection (Simone et al., 1998) or repeated skin applications (Nolano et al., 1999). Simone and colleagues (1998) hypothesized that capsaicin produced a gradual and limited dying back of terminal nerve fibres in the epidermal layers, a common pattern of degeneration in clinical neuropathies, such as diabetic neuropathy (Kennedy et al., 1996) and HIV-associated neuropathy (McCarthy et al., 1995). In recent years, this model has been used to study nerve regeneration in a trial of a pharmacological compound with neuroregenerative properties (Polydefkis et al., 2006), and to study the rate of nerve regeneration in HIV-infected patients (Hahn et al., 2007).

4.2. Mismatch in the relationship between IENFd and LEPs

The mismatch in the relationship between the density of regenerated IENFs (immunostained with PGP9.5) and evoked sensation after capsaicin application has been reported by several investigators (Nolano et al., 1999; Simone et al., 1998; Khalili et al., 2001; Malmberg et al., 2004). Here we show that this was also the case with CO_2 laser stimulation and more specifically with the late-LEPs (i.e., Aδ-nociceptor related brain responses). Within this context, two issues shall be considered: (a) the persistence of heat detection at Day 1 despite the near absence of late-LEPs and IENFs and (b) the fast recovery of late-LEPs despite the slow rate of recovery of morphological signs of regeneration (PGP9.5 and TRPV1 IENFs).

We speculate that at some early post-capsaicin stage, ultra-late-LEPs (i.e., C-nociceptor related brain responses) could be recorded assuming an equal sensitivity to the neurotoxic effect of capsaicin and recalling that the distribution density of Aδ-nociceptors is less than that of C-nociceptors. Indeed, it is a well-established fact that ultra-late-LEPs are only observed in the absence of Aδ-nociceptor activation (Plaghki and Mouraux, 2003). However, this was never the case although, at Day 1, we were able to determine a C-fibre-related threshold and 23% of high intensity stimuli were still detected, but with less than 3% in a time window compatible with Aδ-related activation (Table 3). This may indicate that the very few remaining functional C-fibres at Day 1 allowed detecting heat stimuli, but that the afferent barrage was not strong and/or synchronous enough to allow extraction of C-related brain responses to emerge from the ongoing EEG activity. Indeed, as supported by intraneural microstimulation studies in humans (Ochoa and Torebjörk, 1989) and by laser stimulation with tiny ($\leqslant$0.15 mm^2) beams (Bragard et al., 1996), relatively few epidermal nerve fibres are needed for sensory detection.

It may also be possible that the early evoked sensations and LEPs arise from nociceptors located on sub-epidermal neural plexus. Indeed, density of sub-epidermal neural structures revealed by GAP-43 staining increases in the early stages of regeneration and is in phase with the recovery of LEPs. Although we cannot rule out this possibility with the large thermal probes and long duration (tens of seconds) heat ramps, it seems unlikely that sub-epidermal heat sensitive nerve endings can significantly contribute to these responses. As a fact, the short laser stimulus (50 ms) and the very low transparency of skin for CO_2 laser radiation (Bromm and Treede, 1983) confine the thermal energy to a tiny volume of epidermal tissue as attested by the very small amount of energy needed to bring the skin temperature above the activation threshold of polymodal nociceptors. Laser sources emitting in the short IR range of wavelengths (e.g., 1.34 μm for the Nd:YAP-laser) may possibly activate nociceptors into the sub-epidermal layers since skin has higher transmittance. These lasers have to heat up a much larger volume of tissue to exceed AMH thresholds, as attested by the more than tenfold increase in energy density (Leandri et al., 2006; Perchet et al., 2008; Iannetti et al., 2006). If true, the CO_2 laser radiation may be considered as a better choice to explore more selectively the functional state of the free nerve endings in the epidermal layer. For instance, using a Nd:YAP-laser stimulator, Devigili et al. (2008) could not find any significant differences in latency and amplitude of late and ultra-late-LEPs in a subset of 10 patients with small-fibre neuropathy.

Finally, Polydefkis et al. (2004) ruled out the possibility that the regenerative responses were the result of collateral sprouting emanating from the capsaicin treatment border, which according Rajan et al. (2003) is much slower and incomplete as compared to regenerative sprouting. Indeed, our data also showed that the early stage of regeneration was characterized by an increase in transbasal membrane nerve fibres followed at a later stage by branching of those fibres and a concomitant increase in IENFd. Furthermore, they rigorously addressed also the possibility that capsaicin-induced loss of IENFs staining might not represent sensory fibre loss, but rather loss of PGP9.5 staining, and provided clear evidence that topical capsaicin induces a loss of IENFs by degeneration.

However, one may speculate that the functional vs morphological mismatch might only be apparent. Indeed, PGP9.5, a component of the ubiquitin system involved in protein degradation and turnover of axonal cytoskeletal components (Fraile et al., 1996) is transported exclusively with the slow axonal component b. The long time needed for these proteins to reach distant axonal locations may explain why PGP9.5 appears delayed by several weeks as compared to the functional recovery. The similar time course of TRPV1 immunoreactivity may suggest that expression of TRPV1 receptors is delayed by a similar mechanism, in addition to the disconnection of sensory nerve terminals from their major source of NGF in the basal epidermis (Anand et al., 1996); NGF is known to regulate TRPV1 expression (Winston et al., 2001). As reported by Hiura and Ishizuka (1989), capsaicin-induced dysfunction of the endoplasmic reticulum (ER) can result in improper synthesis and trafficking of TRPV1. Like PGP9.5, TRPV1 is also believed to be transported through the slow axonal component b towards the periphery (Brown, 2000). Capsaicin may delay or impair axonal transport by disassembly of dynamic microtubules necessary for the axonal flow (Goswami et al., 2006). Further, the absence of TRPV1 immunoreactivity may not preclude detection of noxious thermal stimuli, since they may be transduced through multiple molecular pathways, only some of which may involve TRPV1 (Caterina et al., 2000). However, the close temporal relationship between the early recovery of LEPs and the increase in SEFs immunostained for GAP-43, which is transported axonally, indicates a mechanism related to regenerating nerve fibres, and that PGP9.5 and TRPV1 expression may be relatively decreased in early regeneration. Injury to axons stimulates increase in the synthesis of GAP-43, which utilises fast axonal transport to the growth cone (Benowitz and Routtenberg, 1997; Bisby and Tetzlaff, 1992; Denny, 2006).

5. General conclusions

The method consisting of three consecutive days of 0.075% topical capsaicin cream application was successful in mimicking a "reversible small-fibre neuropathy". Comparative assessment of CO_2 laser stimulation and IENFd with the standard PGP9.5 and also TRPV1 immunostaining revealed a mismatch at approximately 12 days post-capsaicin, with a full functional but not morphological recovery – the IENF fibres recovered by only 50% after 54 days. In contrast, the GAP-43 fibres in the sub-epidermis closely followed the amplitudes recorded with CO_2 laser stimulation. We speculated that the mismatch might result from the delay in expression of PGP9.5 and TRPV1 in regenerating IENFs otherwise sensitive to thermal stimuli, which are marked by GAP-43 immunostaining. Consequently, interpreting skin biopsies on the basis of PGP9.5

immunostaining alone, as is conventional, may miss significant diagnostic or prognostic information regarding regenerating fibres if other approaches are neglected, e.g. LEPs or GAP-43 staining.

We recommend CO_2 laser stimulation for assessing IENF function in experimentally induced small-fibre neuropathies, as it is a non-invasive and potentially very sensitive method. In combination with dermal capsaicin application, the CO_2 laser may also be useful in studying degenerating and regenerating sub-epidermal nerve fibres in human volunteer models, and in longitudinal studies of patients with neuropathic disorders.

Conflict of interest

The authors declare that they have no competing interests but M.T., J.S. and T.M. are employees of Johnson and Johnson. M.R. was supported by a grant from Johnson and Johnson.

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