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Dermal nerve fibre and mast cell density, and proximity of mast cells to nerve fibres in the skin of patients with complex regional pain syndrome

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Abstract

An interaction between cutaneous nerves and mast cells may contribute to pain in complex regional pain syndrome (CRPS). To explore this, we investigated the density of dermal nerve fibres, and the density and proximity of mast cells to nerve fibres, in skin biopsies obtained from the affected and unaffected limbs of 57 patients with CRPS and 28 site-matched healthy controls. The percentage of the dermis stained by the pan-neuronal marker protein gene-product 9.5 was lower in the affected limb of patients than in controls (0.12 ± 0.01% versus 0.22 ± 0.04%, $p<0.05$), indicating a reduction in dermal nerve fibre density. This parameter did not correlate with CRPS duration. However, it was lower in the affected than unaffected limb of patients with warm CRPS. Dermal mast cell numbers were similar in patients and controls, but the percentage of mast cells less than 5 μm from nerve fibres was significantly lower in the affected and unaffected limbs of patients than in controls (16.8 ± 1.7%, 16.5 ± 1.7% and 31.4 ± 2.3% respectively, $p<0.05$). We confirm previous findings of a mild neuropathy in CRPS. Our findings suggest that this either develops very early after injury or precedes CRPS onset. Loss of dermal nerve fibres in CRPS might result in loss of chemotactic signals, thus halting mast cell migration towards surviving nerve fibres. Failure of normal nerve fibre-mast cell interactions could contribute to the pathophysiology of CRPS.

Key words: complex regional pain syndrome; mast cells; dermal nerve fibre density; inflammation

Introduction

Complex regional pain syndrome (CRPS) usually begins after a fracture or soft tissue injury (CRPS I) but may also be triggered by major peripheral nerve trunk injury (CRPS II). Shortly after injury, the affected limb often is red, swollen and warm, in association with raised levels of pro-inflammatory mediators [6,24] and an increased presence of cutaneous mast cells [5,19,40]. Inflammatory signs generally subside within the first year of CRPS, but later on the affected limb may become cold and blue [6]. Skin biopsy studies indicate that CRPS is associated with cutaneous small fibre pathology [23,39,42], but whether this precedes the injury, is caused by injury-associated inflammatory changes or is part of a later neurodegenerative process is unknown.

In health and disease, mast cells interact with nerves, blood vessels and other immune cells to modulate neuro-immune responses [1,15,33]. On encountering allergens or pathogens, mast cells secrete a wide range of enzymes, chemo-attractants, immuno-modulators, vasoactive compounds, inflammatory mediators and growth factors [18], consequently generating bi-directional paracrine interactions with nerve fibres [1]. Both in patients with early CRPS and in a rodent tibia fracture model of CRPS, total dermal mast cell numbers and levels of mast cell tryptase are raised on the affected side [5,19,29]. Further, in the CRPS rodent model [29], and in inflammatory skin diseases such as atopic dermatitis [47] and psoriasis [36,37], mast cells are situated closer to nerve fibres in affected- than unaffected skin, suggesting the possibility of neuronal sensitization. That such an effect might contribute to the pathology of primary human chronic pain conditions has been suggested by experiments in irritable bowel syndrome where the 'closeness' of mast cells to intestinal mucosal sensory nerve endings correlates directly with pain intensity [3].

To our knowledge, the proximity of dermal mast cells to nerve fibres has not been examined in patients with CRPS. We hypothesized that mast cells would congregate around dermal nerve fibres in CRPS-affected skin [1].

Methods

Participants

Patients who met research diagnostic criteria for CRPS [16] and who were at least 18 years old were recruited from a small private pain medicine centre in Perth, Western Australia. In addition, healthy pain-free controls were recruited from university staff and students, and from friends and spouses of patients. This study formed part of a larger investigation that involved injecting chemicals into the skin (manuscript in preparation). Therefore, patients were excluded if they were pregnant or breastfeeding; if they had a medical condition that affected their heart, blood vessels, skin, liver or

kidneys that required regular treatment with medication; if they had a known sensitivity to adrenergic drugs; or if they had severe hypertension, arrhythmias, hyperthyroidism or hyperglycaemia. Patients with injuries to more than one limb were also excluded. Ethics approval was granted by the Murdoch University Research Ethics Committee, and informed, written consent was obtained from all participants.

Assessment of pain

In an initial standard clinical interview, patients were asked to rate their current pain intensity on an 11-point numeric rating scale between 0 (no pain) and 10 (extremely painful), and to describe whether their pain was associated with sensations of aching, stabbing, throbbing, burning, numbness, or pins-and-needles (paraesthesiae) (each answered either yes, or no).

Skin temperature

Skin temperature was measured from the dorsal surface of the first phalanx of each digit on the affected and contralateral limb of CRPS patients with an infrared thermometer (Tempett IR Thermometer, Somedic Sales AB, Sweden). Patients were allocated to the warm CRPS subtype if digits on the affected limb were at least 1^{o}C warmer than on the contralateral limb, and to the cold CRPS subtype if digits on the affected limb were at least 1^{o}C cooler than on the contralateral limb [6,17]. The other patients were allocated to an "undetermined" subtype. Skin temperature was measured after participants had acclimatized for at least 45 minutes in a temperature-controlled room maintained between 21^{o}C and 24^{o}C.

Sample preparation

Under local anaesthesia, two 3 mm punch skin biopsies were collected from each CRPS patient; one from a painful site in the hand or foot on the injured side and the other from a mirror-image site in the contralateral limb. In patients with a history of nerve injury, biopsies were taken from a site that was sensitive to stimulation. Biopsies from healthy volunteers were obtained from similar sites to CRPS-affected sites. The biopsies were fixed in Zamboni's solution, processed and embedded in paraffin, and cut at 10 μm for histochemical staining of mast cells in the dermis. Studies were restricted to these thin sections to ensure full antibody penetration through the paraffin-embedded tissue.

Nerve fibre and mast cell counts

Prior to staining, paraffin was removed from tissue sections in xylene and sections were then rehydrated in a descending series of ethanol concentrations. Sections were rinsed in running water for 30 minutes and incubated in 0.1M phosphate buffered saline (PBS) for 5 minutes. For antigen retrieval, sections were incubated in 1 mg/ml trypsin for 30 minutes at 37°C, followed by cold 0.1M phosphate buffer (2x2 minutes) and PBS-triton-X (0.5%, 5 minutes). Next, sections were incubated in blocking solution (10% donkey serum with PBS) for 2 hours at room temperature, followed by primary antibodies in a humidified chamber at 4°C for 48 hours: rabbit anti-human protein gene-product 9.5 (PGP9.5; Bio-rad AbD Serotec, North Carolina, USA), a pan-neuronal marker that identifies all cutaneous nerves; and monoclonal mouse, anti-human mast cell tryptase clone AA1 (DAKO, Glostrup, Denmark). Sections were then rinsed in PBS (3x15 minutes) and incubated in secondary antibodies; Cy3™ –conjugated donkey anti-rabbit and AlexaFluor®-488 conjugated donkey anti-mouse (Jackson Laboratories, West Grove, Pennsylvania, USA) for 2 hours at room temperature. Sections were rinsed with PBS (3x15 minutes), mounted with ProLong Gold anti-fade with DAPI reagent (Molecular Probes by Life Technologies, Eugene, Oregon, USA), and stored at 4°C.

Substance P immunohistochemistry

In separate assays, paraffin was removed from tissue sections in xylene and sections were rehydrated in a descending series of ethanol concentrations and rinsed in running water for 10 minutes. After antigen retrieval for 2 minutes in boiling sodium citrate buffer at pH 6.0, sections were incubated in PBS (5 minutes) and PBS-triton-X (0.5%, 5 minutes). Next, sections were incubated in blocking solution (10% donkey serum with Tris buffered saline with 0.5% Tween 20) for 2 hours at room temperature. Sections were then incubated in primary antibodies in a humidified chamber at 4°C for 48 hours: monoclonal rabbit anti-human protein gene-product 9.5 (PGP9.5; dilution 1:500; Abcam, Cambridge, United Kingdom), polyclonal guinea pig anti-substance P (SP; 1:100 dilution; Abcam Cambridge, United Kingdom) and monoclonal mouse, anti-human mast cell tryptase clone AA1 (dilution 1: 4000; DAKO, Glostrup, Denmark). Sections were then rinsed in PBS (3x15 minutes) and incubated in secondary antibodies: Cy3TM –conjugated donkey anti-rabbit and AlexaFluor®-488 conjugated donkey anti-guinea pig (Jackson Laboratories, West Grove, Pennsylvania, USA) and AlexaFluor®-647 donkey anti-mouse for 2 hours at room temperature. Sections were rinsed with PBS (3x15 minutes), mounted with ProLong Gold anti-fade with DAPI reagent (Molecular Probes by Life Technologies, Eugene, Oregon, USA), and stored at 4°C.

Specificity of antibodies

The specificity of the mast cell tryptase antibody has been validated in Western blot, indirect ELISA and antibody-binding studies [49], and the specificity of the PGP9.5 antibody has been validated by high-resolution two-dimensional polyacrylamide gel electrophoresis and Western blot [20,26]. The PGP9.5 antibody used in the substance P analysis has been validated in knockout studies to detect human PGP9.5 in flow cytometry, immunocytochemistry, immunohistochemistry and Western Blot assays (manufacturer's datasheet, Abcam, Cambridge, United Kingdom). The specificity of the substance P antibody was validated within our laboratory with a peptide block, corresponding to amino acids 1-11 of rat substance P (Abcam, Cambridge, United Kingdom).

Image collection and quantification

Images of dermal nerve fibres and mast cells were collected using a Nikon Eclipse Ti multiphoton confocal microscope. Each fluorescent label was imaged sequentially at the appropriate excitation and emission spectra to prevent bleed-through between channels. Three to five adjacent microscopic fields were selected across each section to encompass the dermis bordering the dermal-epidermal junction to a depth of 150-200 μm. For each microscopic field, several images were taken at different focal planes (z-stack), with a step-size of 1 μm and a magnification of 40x, numerical aperture=0.95. For the subsequent analysis, each image in the z-stack was merged into one horizontal projection at maximum intensity (Figure 1). ImageJ software (version 1.50d, National Institutes of Health, USA) was used to analyse nerve fibre densities, total mast cell numbers and proximity of nerve fibres to mast cells, with the operator blinded to the sample group.

As mast cells are located in the dermis and not the epidermis, we were interested in quantifying dermal nerve fibre density (i.e., the percentage of the selected dermal area stained by PGP9.5) [2,35]. For each section, the dermal area (traced manually from the epidermal-dermal junction to a depth of 150 μm) was expressed as the number of pixels within the traced area. Background nonspecific fluorescence was excluded by adjusting the brightness threshold to highlight large- and small-diameter nerve fibres stained by PGP9.5 or substance P. All pixels within the traced dermal area with brightness equal to or higher than the pre-set threshold for PGP9.5 or substance P staining were marked as immuno-positive (supplementary Figure 1). Dermal nerve fibre density was then calculated as the percentage of immuno-positive pixels within the traced dermal area. Substance P fibres were expressed as a percentage of total PGP9.5 staining.

The number of mast cells (defined as a nucleated profile immunoreactive to mast cell tryptase antibody) within the dermal area was counted manually, and was expressed as the number of mast cells per mm^2. The distance between each mast cell and the closest nerve fibre within the plane of

the section was measured, and the number of mast cells in close proximity (<5 μm) to nerve fibres stained by substance P and/or PGP9.5 was counted [3] and expressed as a percentage of the total mast cell number.

Statistics

Data were pooled for the upper and lower limbs as no differences between the limbs were identified in preliminary analyses for any of the parameters of interest (nerve fibre density, mast cell density, or percentage of mast cells in close proximity to nerve fibres). Differences between the CRPS-affected limb and controls, and between the unaffected limb of patients and controls, were investigated in independent-samples t-tests, with Bonferroni correction for multiple contrasts. Differences between the CRPS-affected and contralateral limbs were further investigated in relation to i) diagnostic sub-groups (CRPS I, CRPS II); ii) disease duration (classified as up to 12 months [acute], between 13 and 36 months [intermediate], and longer than 36 months [chronic]); and iii) asymmetry of limb temperature (the warm, cold and undetermined subtypes). Each between-groups factor was investigated separately because of sample size limitations. SPSS version 24 was used for statistical analyses, and the criterion of statistical significance was $p < 0.05$.

Results

Thirty-nine patients met research diagnostic criteria for CRPS I [16] and 18 patients met criteria for probable or definite peripheral nerve injury [13] and CRPS II [16] (Table 1). Skin biopsies were also obtained from 28 pain-free healthy volunteers of similar age and sex distribution to patients (mean age 45.7 ± 15.9 years; 18 females). By-and-large, demographic details and pain characteristics were similar within the various CRPS subgroups. However, females were more likely to have CRPS I than CRPS II, and warm than cold CRPS (Table 1). In addition, sensations of numbness were reported by a smaller percentage of patients with chronic than intermediate or acute CRPS (Table 1).

Nerve fibre density in the CRPS-affected limb correlated with nerve fibre density in the unaffected limb [$r(53) = 0.40$, $p<0.01$]. Nerve fibre density was lower in the CRPS-affected limb than in controls (Figure 1 and Table 2, $p<0.05$). Substance P staining within PGP9.5-labelled nerve fibres (Figure 2) was low, and was similar in patients and controls (Table 2).

Mast cell density in the CRPS-affected limb correlated with mast cell density in the unaffected limb [$r(54) = 0.51$, $p<0.001$]. Mast cell density was lower in patients than controls, but the significance of this trend was lost after Bonferroni correction for multiple contrasts (Table 2). Mast cell-nerve fibre proximity in the CRPS-affected limb correlated with mast cell-nerve fibre proximity in the unaffected

limb [r(53) = 0.49, p<0.001]. The percentage of mast cells in close proximity to nerve fibres was significantly smaller both in the affected and unaffected skin of patients than controls (Table 2, p<0.001). Only a small percentage of mast cells were found within 5 μm of nerve fibres stained by substance P; this percentage was similar in patients and controls (Table 2).

In general, dermal nerve fibre density, substance P staining within PGP9.5-labelled nerve fibres, dermal mast cell density, and the percentage of mast cells in close proximity to nerve fibres were similar in patients with CRPS I and CRPS II, and in patients with acute, intermediate and chronic CRPS (not shown). However, substance P staining within PGP9.5-labelled nerve fibres was greater bilaterally in patients with CRPS II than CRPS I (7.2 ± 1.0% versus 4.3 ± 0.7%, p<0.05). Six patients reported mild pain in the limb contralateral to the site of injury. Dermal nerve fibre density, dermal mast cell density, and the percentage of mast cells in close proximity to nerve fibres were similar in the contralateral limb of these patients and the contralateral limb of patients with unilateral limb pain. In the group as a whole, neither dermal nerve fibre density, substance P staining within PGP9.5-labelled nerve fibres nor mast cell-nerve fibre proximity correlated with symptom duration (not shown). However, as expected in patients whose symptoms had persisted for less than a year (n=21), mast cell density in affected skin correlated inversely with disease duration [r(18) = -0.48, p<0.05] (Figure 3A). Mast cell density was unrelated to symptom duration in patients with intermediate CRPS (Figure 3B), but increased in line with symptom duration in patients with chronic CRPS [r(16) = 0.54, p<0.05] (Figure 3C). In contrast, nerve fibre density was unrelated to symptom duration in acute, intermediate or chronic CRPS (Figure 3D-F).

We found previously that mast cell density was elevated in affected skin during the first three months of CRPS [5]. In the present study, skin biopsies were obtained from only three patients within this timeframe. In these patients, dermal mast cell density again was greater in affected skin than in controls (245 ± 23 mast cells/mm^2 versus 156 ± 10 mast cells/mm^2, Mann-Whitney U test p<0.05). Nevertheless, the percentage of mast cells in close proximity to nerve fibres was smaller in the affected and unaffected skin of even these patients (31 ± 2% in controls versus 17 ± 2% and 14 ± 5% respectively, Mann-Whitney U test p<0.05).

The affected limb was at least 1^oC warmer than the contralateral limb in ten patients (warm CRPS subtype, eight with CRPS I), and cooler than the contralateral limb by this margin in another 13 patients (cold CRPS subtype, six with CRPS I). Limb temperatures differed from each other by less than 1^oC in another 34 patients (CRPS subtype undetermined, 25 with CRPS I). There was no clear association between the thermal subtypes and duration categories. Dermal nerve fibre density was lower in the affected than unaffected limb of patients with warm CRPS [Side x CRPS subtype

interaction $F(2,52) = 3.83$, $p<0.05$] (Figure 4); in contrast, dermal nerve fibre density was lower in both limbs of patients with symmetrical limb temperatures than controls (Figure 4). Dermal mast cell density and the percentage of mast cells in close proximity to nerve fibres were similar in the affected and unaffected limbs of patients in all three thermal subtypes (not shown).

The density of mast cells and/or nerve fibres in the dermis might influence their proximity; for example, the higher the density of mast cells, the greater the likelihood of coincidental proximity to nerve fibres. Therefore, associations between mast cell density, nerve fibre density and nerve fibre-mast cell proximity were investigated in patients and controls using Pearson's correlation coefficient. The percentage of mast cells in close proximity to nerve fibres increased in proportion to dermal nerve fibre density in all groups (Figure 5A-5C). Nevertheless, the slope of this relationship (i.e., the increase in dermal nerve fibre density in relation to an increase in the percentage of closely apposed mast cells) was steeper in controls than in CRPS-affected and unaffected skin (Table 3). In CRPS patients, the percentage of mast cells in close proximity to nerve fibres increased in proportion to dermal mast cell density; however, in controls, this trend was reversed (Figure 5D-5F and Table 3).

In exploratory analyses, dermal nerve fibre density in the affected limb was lower in patients who described their pain as burning ($N = 43$) than in the remainder of patients ($N = 12$) [0.11 ± 0.01 versus 0.16 ± 0.03 % of dermal area, $t(54) = 2.04$, $p<0.05$ without Bonferroni correction]. However, neither dermal nerve fibre density, substance P staining within PGP9.5-labelled nerve fibres, mast cell density nor mast cell-nerve fibre proximity in the affected limb were related to any other pain descriptor or to current pain intensity.

Discussion

Cutaneous nerve fibres that follow the dermal-epidermal border send terminal 'neurite' twigs into the epidermis. The density of these neurites is reduced in affected skin [23,39] or bilaterally [42] in at least a subgroup of CRPS patients. In the present study, the density of the dermal nerve fibres that supply these neurites was lower in CRPS-affected skin than in controls, and also was reduced on the uninjured side in some patients. In addition, the proportion of mast cells in close proximity to dermal nerve fibres was lower in CRPS patients than controls, not only in the affected limb but also in the contralateral uninjured limb. Together, these findings suggest that CRPS is associated with dermal neuropathology, potentially disrupting neural interactions with dermal mast cell populations.

In previous studies, intra-epidermal nerve fibre density was examined almost exclusively in patients with longstanding CRPS [23,39,42] whereas our sample contained patients with various disease durations, including relatively short periods. Dermal nerve fibre density in the affected limb was

unrelated to disease duration or CRPS subtype, suggesting that dermal neuropathology develops soon after injury or perhaps even *precedes* limb trauma; if, so, this could form part of a CRPS-prone phenotype [4,42]. Warm CRPS often begins earlier in the clinical presentation than cold CRPS, and is associated more strongly than cold CRPS with signs of inflammation [6]. In the present study, dermal nerve fibre density was lower in the affected than contralateral limb of patients with warm CRPS, but was reduced bilaterally in most other patients. While this might suggest a progressive inflammation-driven decline in nerve fibre density [6], longitudinal studies would be required to confirm this (e.g., studying patients before and after elective surgery).

Our sample included a relatively high proportion of patients with CRPS II (32% compared with only 15% in a recent large cross-sectional study [14]), perhaps reflecting referral biases in the small pain medicine practice where our patients were sourced. Patients were diagnosed with CRPS II based on evidence of major peripheral nerve trauma; however, skin samples were obtained from a site with at least partially-preserved sensation. Nerve fibre density in the papillary dermis was reduced in the painful and contralateral uninjured limb of these patients, and resembled dermal nerve fibre density in patients with CRPS I. Thus, it seems plausible that similar mechanisms disrupted dermal nerve fibre density in CRPS I and CRPS II. Despite different triggers, symptom profiles are similar in both forms of CRPS [9,14], suggesting that mechanisms which contribute to chronicity are shared. Loss of dermal nerve fibres may be one such shared mechanism.

Trauma initially leads to attraction and migration of mast cells into the dermis, followed by their degranulation [25,29]. Consequently, during the first three months of CRPS, mast cell numbers and inflammatory cytokines are elevated in the affected dermis [5]; this may promote a mast cell-neuron feedback loop that contributes to nociceptor sensitization and pain [1,19,25,29]. Even so, we found a *smaller* proportion of mast cells in close apposition to dermal nerve fibres bilaterally across the full spectrum of CRPS than in controls. Together with a bilateral decline in dermal nerve fibre density, this may represent failure of nerve-mast cell interactions in CRPS. CRPS is associated with bilateral hyperalgesia, heightened neurogenic inflammation, and up-regulated α_1-adrenoceptor expression [12,27,28,48]; such bilaterality might be caused by systemic pathology and/or by spinal disturbances that evoke mirror-image changes after limb trauma [38,43]. Whether such changes account for the bilateral reduction in nerve fibre-mast cell proximity in CRPS, or whether this reflects extensive bilateral degranulation of mast cells, requires further investigation.

Mast cells congregate around blood vessels, hair follicles and nerve fibres in the skin [7]. Neural production of chemo-attractants [33] might explain why the percentage of mast cells in close proximity to nerve fibres was strongly associated with dermal nerve fibre density both in patients

and controls (i.e., the greater the nerve fibre density, the stronger the chemo-attraction). We found no evidence that heightened production of substance P in CRPS [45] altered mast cell distributions [29], perhaps because most of our patients were studied after acute inflammatory responses to injury had subsided. However, other neural sources of chemo-attraction (e.g., calcitonin gene-related peptide [31,44]) might stimulate mast cell recruitment. The percentage of mast cells in close proximity to nerve fibres increased in line with mast cell density in patients but decreased in controls, possibly because sources of chemo-attraction were more diverse in controls than patients. For example, in controls, strong non-neural chemotactic signals might have attracted mast cells from the bloodstream or could have drawn resident mast cells away from dermal nerve fibres. Nonetheless, the percentage of mast cells in close proximity to nerve fibres was, on average, half as great in patients than controls, suggesting that nerve fibre-mast cell contacts were compromised. This might form part of a broader disruption of neuro-immune interactions, as Langerhans cells (whose antigen-presenting properties are stimulated by neuropeptides) are more numerous in affected than unaffected skin in the CRPS tibia fracture model and in acute CRPS [30]; however, this reverses in patients with longstanding CRPS [40].

Mast cells are involved not only in innate and acquired immunity but also in the inflammation, proliferation and remodeling phases of wound healing [7]. They synthesize nerve growth factor, a neurotrophin that regulates neuronal survival and neurite outgrowth, thus modulating inflammatory and immune responses [46]. In turn, nerve fibres attract mast cells and influence their activity via neuropeptides [11,29,33,34,50], thus effectively attracting a source of nerve growth factor. Mechanisms that inhibit neurite outgrowth in patients with CRPS, and that cause inflammation to persist beyond the period required for tissue repair, are unknown, but disruption of nerve growth factor synthesis or release potentially plays a role. Intriguingly, stimulation of α_1-adrenoceptors hinders the expression of nerve growth factor in cardiac myocytes [41]. Thus, we speculate that stimulation of up-regulated α_1-adrenoceptors in CRPS-affected skin [8,10,12] inhibits the production of nerve growth factor, hence impeding neurite outgrowth and disrupting their alliance with mast cells. Loss of dermal nerve fibres might also result in loss of chemotactic signals, thereby halting mast cell migration toward surviving neurites.

A limitation of this project was the small number of patients available for study with very early CRPS. In the three patients with <3 months disease duration, dermal mast cell density was greater in affected skin than in controls, replicating previous findings [5]. Interestingly, even in these patients, a smaller proportion of mast cells were closely apposed to dermal nerve fibres than in pain-free controls, implying early disruption of neural-mast cell interactions in CRPS. Mast cell density

increased in line with symptom duration in patients with chronic CRPS but, nevertheless, mast cell-nerve fibre proximity remained low. This contrasts with the distal tibia fracture model of CRPS in rodents [29] and with irritable bowel syndrome in humans [3], where a particularly close interaction between mast cells and nerve fibres plays a major role in inflammation and pain. Thus, it might be expected that early treatment with mast cell activation blockers would inhibit pain in these conditions [29]. Whether additional therapeutic interventions directed at mast cells (e.g., neural-mast cell attractants) would also modify CRPS pain is yet to be explored.

At 10 µm, skin sections were too thin to calculate the number of intra-epidermal or dermal nerve fibres. However, our methods allowed us to quantify the proportion of the papillary dermis stained by the pan-neuronal marker PGP9.5. This index was lower in affected than control skin, consistent with depletion in CRPS of the dermal nerves that supply intra-epidermal neurites. The index was weighted toward large nerve fibres that occupied most area. Still, the proportion of substance P to PGP9.5 staining was similar in patients and controls, suggesting that decreases in the small-diameter nerve fibres containing substance P most likely were similar to decreases in the total nerve fibre population in CRPS.

We found no difference in dermal nerve fibre density between the hands and feet. Although intra-epidermal nerve fibre density generally is higher in upper than lower limbs [21], this might not apply to the distal extremities [22,32]. It is difficult to compare our findings with previous reports because of differences in sample size, fixation, counting methods and nerve populations, as only free nerve endings penetrate the epidermis.

In conclusion, a bilateral reduction in dermal nerve fibre density in CRPS was unrelated to the patients' disease duration, and was accompanied by a bilateral reduction in proximity between surviving nerve fibres and dermal mast cells which were present in low-to-normal numbers. The neural density-reduction and loss of proximity to mast cells was unrelated to the chronicity or diagnostic sub-type of CRPS, or to current pain intensity. Even so, failure of normal neural-mast cell interactions in CRPS might prolong inflammation and delay tissue repair [7], thus impeding pain resolution. Hence, finding out why mast cells fail to congregate closely around dermal nerve fibres in CRPS could suggest new approaches to treatment.

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Figure Legends

Supplementary Figure 1: Calculation of nerve fibre density. A: The region of interest within the dermis was traced manually from the epidermal-dermal junction to a depth of 150 μm (dotted yellow line), and was expressed as the number of pixels within the traced area. B: All pixels within the traced dermal area with brightness equal to or higher than the pre-set threshold for PGP9.5 staining (red) were marked as immuno-positive. Dermal nerve fibre density was defined the percentage of immuno-positive pixels within the traced dermal area. The scale bar = 100 μm.

Figure 1: Examples of nerve fibre and mast cell staining in skin sections from (A) a control; and (B) a CRPS patient. The white dotted lines indicate the dermal-epidermal junction, and the arrows show mast cells (green) in close proximity (< 5 μm) to nerve fibres (red). Cell nuclei are shown in blue. The scale bar = 100 μm.

Figure 2: An example of staining with antibodies to (A) PGP9.5, (B) mast cell tryptase, (C) substance P, and (D) cell nuclei in the upper dermis and epidermis of a CRPS-affected limb. The closely packed cell nuclei in the upper part of the image represent epidermal cells. (E) The white arrows show mast cells (green) that are in close proximity (less than 5 μm) to nerve fibres (red). Substance P is expressed in a subset of PGP9.5-labelled nerve fibres. The nerve fibre stained by substance P is greater than 5 μm away from mast cells. Scale bar =100 μm.

Figure 3: Association between symptom duration and dermal mast cell and nerve fibre density in acute, intermediate and chronic CRPS. In each graph, the solid line represents the line of best fit, and the dotted lines represent the 95% confidence band of the best-fit line.

Figure 4: Dermal nerve fibre density in relation to the CRPS temperature subtype. * Nerve fibre density lower in affected than unaffected skin ($p<0.05$). # Nerve fibre density lower in patients than controls ($p<0.05$). Error bars represent standard errors.

Figure 5: Association between the percentage of mast cells in close proximity to nerve fibres, dermal nerve fibre density and dermal mast cell density. In each graph, the solid line represents the line of best fit, and the dotted lines represent the 95% confidence band of the best-fit line.

Summary

Loss of dermal nerve fibres in CRPS might disrupt neural-mast cell interactions, thereby delaying tissue repair and contributing to chronic inflammation.

Figure 1

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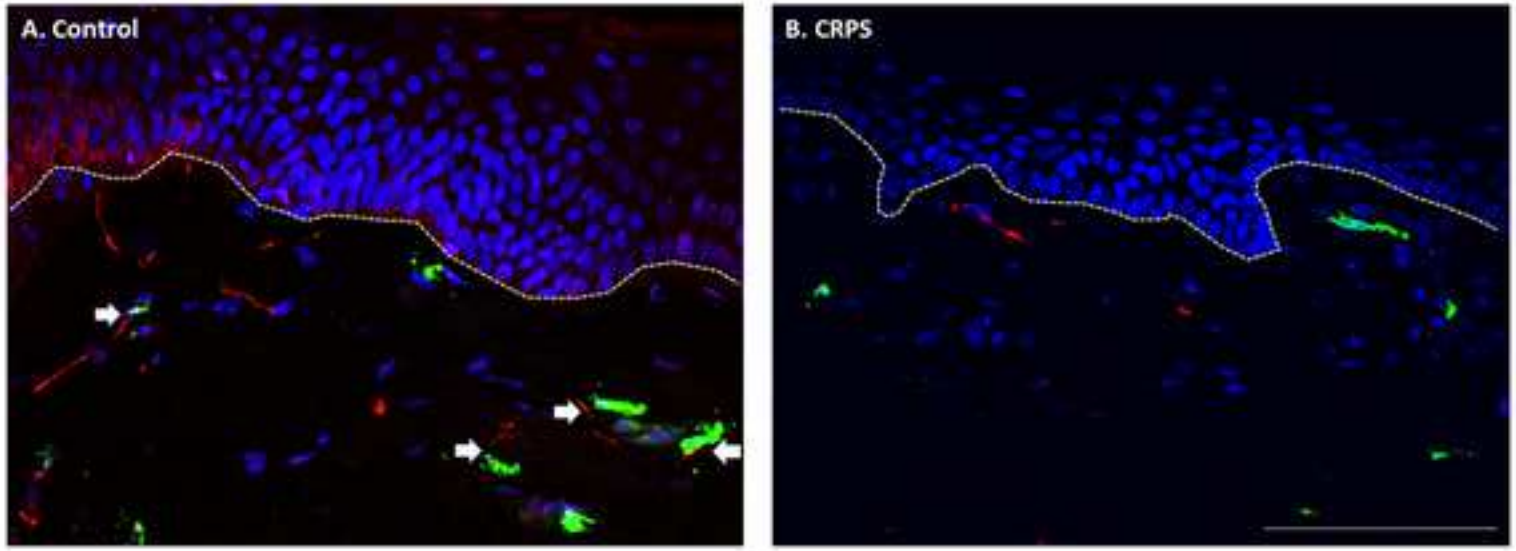

Figure 2

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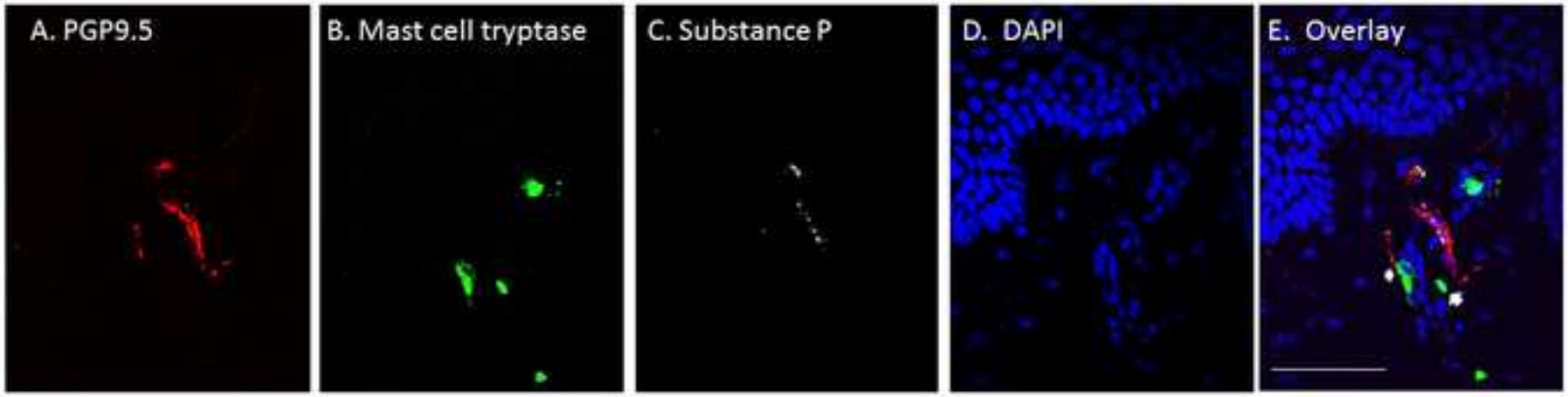

Figure 3

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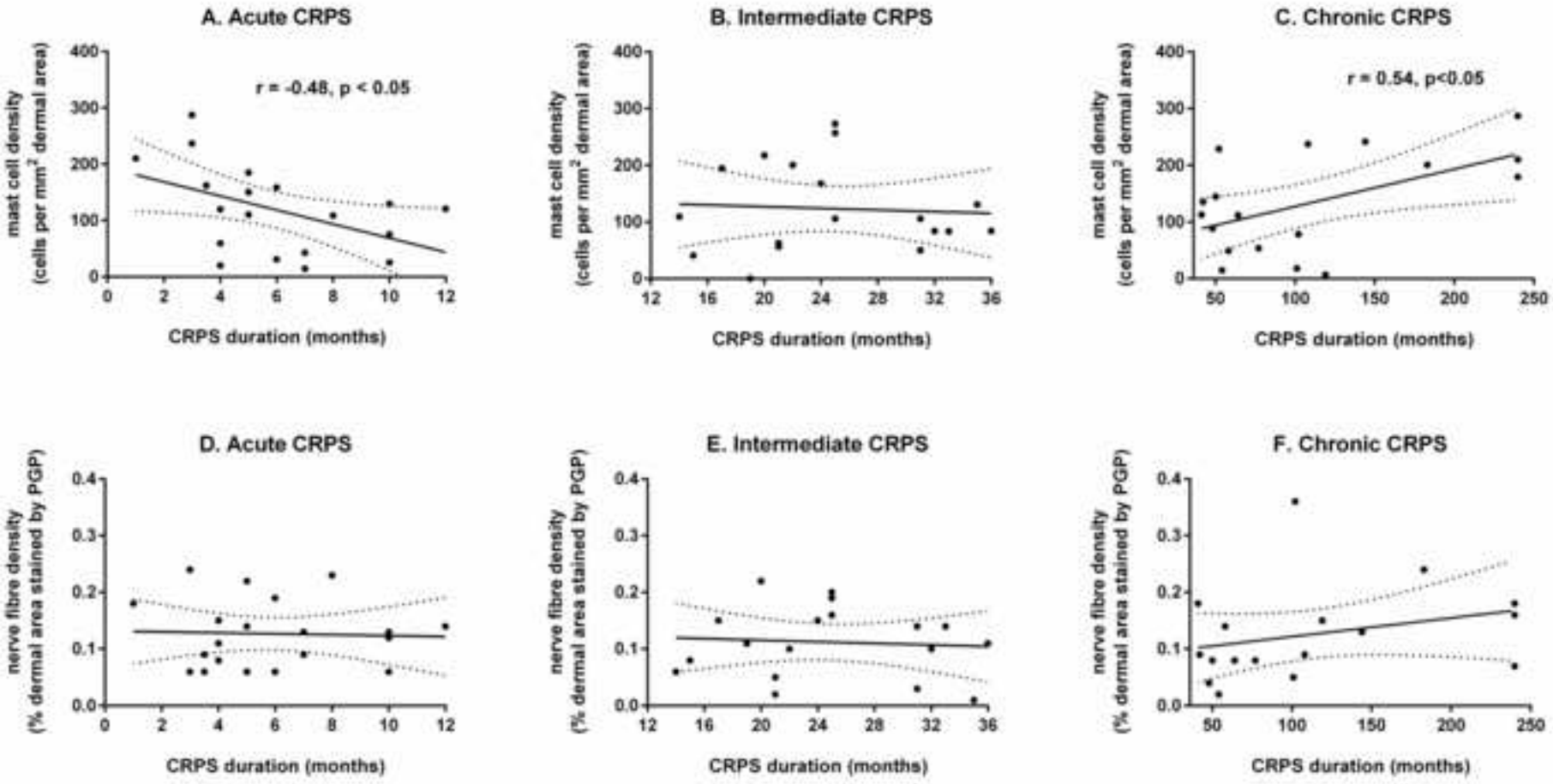

Figure 4

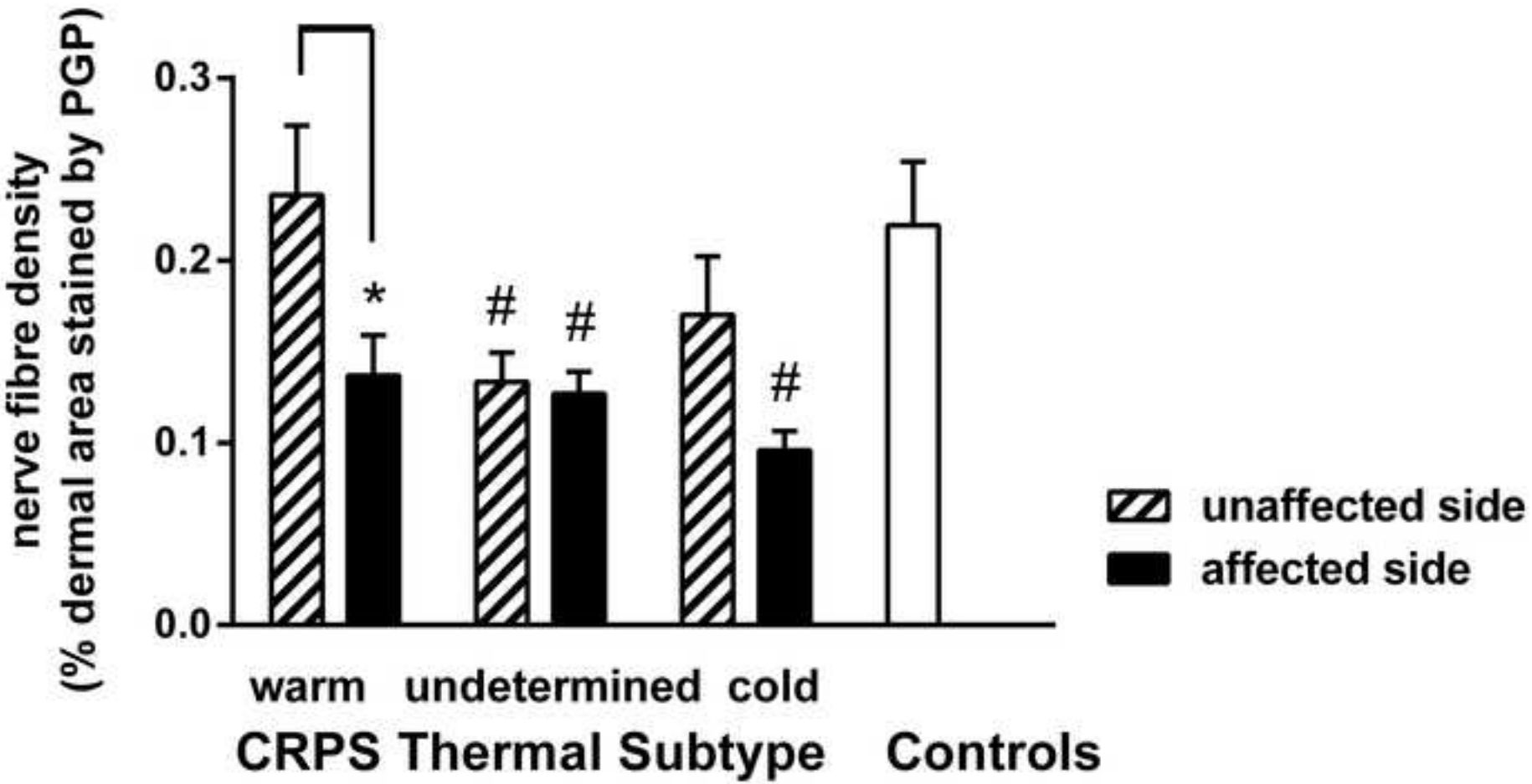

Figure 5

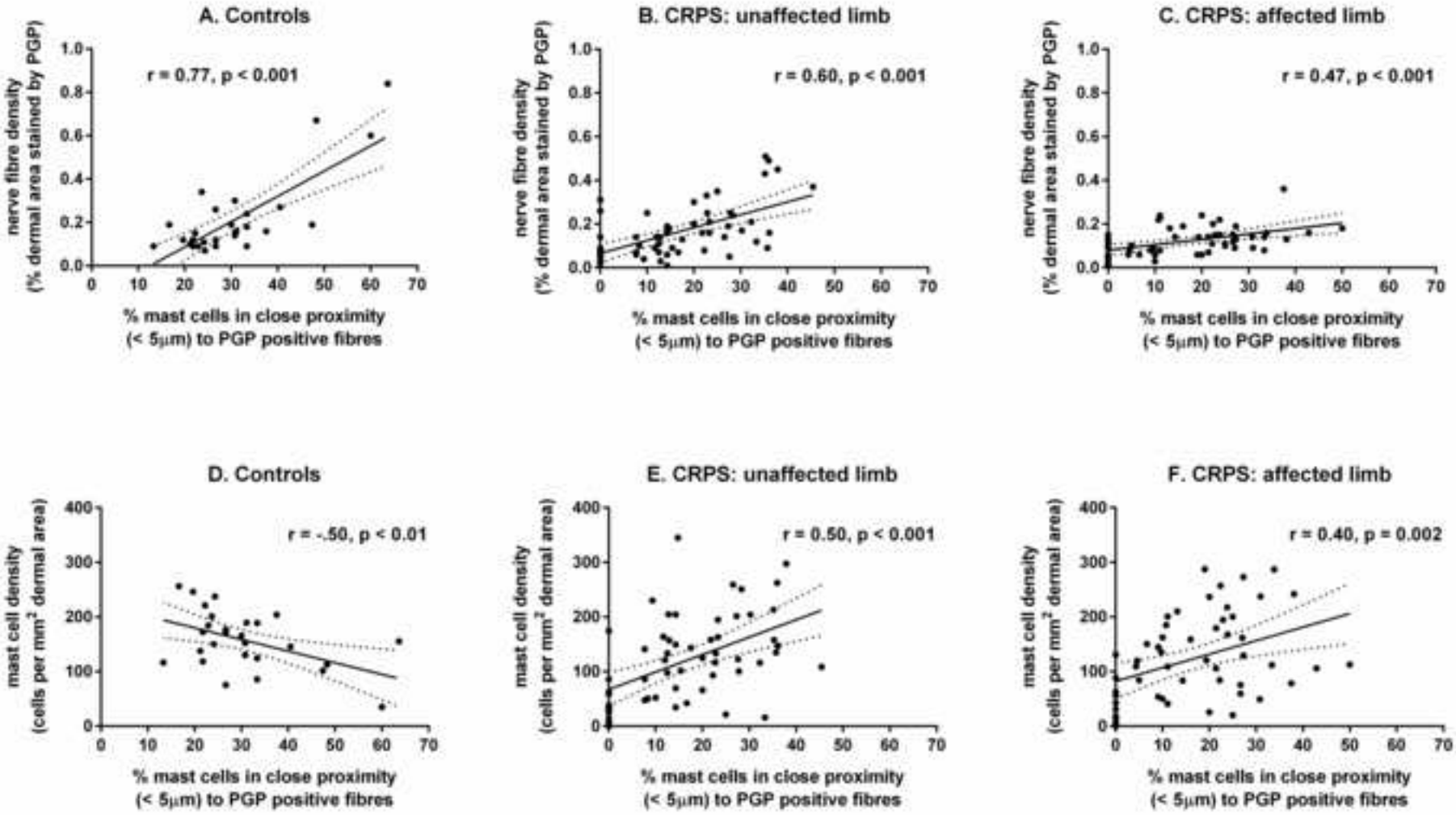

Table 1

Table 1: Demographic details and pain characteristics

	CRPS subtype [a]		Duration category [a]			Thermal subtype [a]		
	CRPS I	CRPS II	acute	intermediate	chronic	cold	undetermined	warm
N	39	18	21	18	18	13	34	10
Females	79% [b]	44%	67%	61%	78%	38%	73%	90% [b]
Upper limb affected (%)	51%	61%	62%	67%	33%	38%	68%	30%
Age ± S.D. (years)	46.0 ± 10.7	47.9 ± 10.7	48.4 ± 9.2	47.1 ± 11.8	44.1 ± 11.1	47.1 ± 9.2	45.5 ± 11.6	49.8 ± 8.9
CRPS duration (months)	41.4 ± 59.2	51.3 ± 62.3	6.1 ± 3.1	24.8 ± 6.8	109.1 ± 71.4	46.9 ± 65.1	43.1 ± 54.2	46.2 ± 76.4
Pain intensity ± S.D. (0-10)	5.0 ± 2.4	4.7 ± 1.8	4.3 ± 2.5	4.9 ± 1.6	5.5 ± 2.3	4.5 ± 1.7	4.8 ± 2.4	5.4 ± 2.2
Aching pain	67%	61%	77%	78%	50%	61%	68%	60%
Stabbing pain	64%	72%	81%	56%	61%	54%	71%	70%
Throbbing pain	54%	44%	53%	50%	50%	31%	59%	50%
Burning pain	79%	78%	76%	78%	83%	85%	76%	80%
Numb sensation	54%	39%	57%	67%	22% [c]	46%	53%	40%
Pins-and-needles (paraesthesiae)	77%	72%	81%	83%	61%	77%	73%	80%

[a] In patients with CRPS II, a peripheral nerve injury had been verified surgically or by a confirmatory test, or a sensory examination and quantitative sensory tests indicated sensory disturbances in an anatomically-plausible nerve distribution given the site and nature of the triggering event [13]. CRPS was considered to be acute if it had persisted for less than 12 months, intermediate if it had persisted for 13-36 months, and chronic if it had persisted for longer than 36 months. Patients were allocated to the warm CRPS subtype if digits on the affected limb were at least 1°C warmer than on the contralateral limb, and to the cold CRPS subtype if digits on the affected limb were at least 1°C cooler than on the contralateral limb [6,17]. The other patients were allocated to an "undetermined" subtype.

[b] More patients with CRPS I than CRPS II were female ($p<0.05$), and more patients with warm than indeterminate or cold CRPS were female ($p<0.05$).

[c] More patients with acute or intermediate than chronic CRPS reported a numb sensation in their affected limb ($p<0.05$).

Table 2

Table 2: Nerve fibre density, mast cell density, and % mast cells < 5 µm from a nerve fibre (mean ± S.E.M.).

	Controls (N = 28)	CRPS (N = 57)	
		Unaffected	Affected
PGP9.5 nerve fibre density (% dermal area)	0.22 ± 0.04	0.16 ± 0.02	0.12 ± 0.01*
Substance P (% PGP9.5 staining)	6.5 ± 0.9	5.0 ± 0.8	5.1 ± 0.7
Mast cell density (cells per mm^2)	155.8 ± 10.1	120.0 ± 10.6 ¶	125.7 ± 10.6 ¶
% mast cells < 5 µm from a PGP9.5-labelled nerve fibre	31.4 ± 2.3	16.5 ± 1.7*	16.8 ± 1.7*
% mast cells < 5 µm from a substance P-labelled nerve fibre	2.6 ± 0.6	2.3 ± 0.4	3.3 ± 0.7

* $p<0.001$ compared with controls (Bonferroni test). ¶ $p<0.05$ compared with controls (without Bonferroni correction).

Table 3

Table 3: Regression line slopes for (i) nerve fibre density; and (ii) mast cell density in relation to the percentage of mast cells < 5 µm from a nerve fibre

	Slope ± S.E.M. in relation to the percentage of mast cells < 5 µm from nerve fibres		
	Controls (N = 28)	**CRPS (N = 57)**	
		Unaffected	**Affected**
Nerve fibre density (% PGP9.5 staining)	0.0117 ± 0.0019	0.0059 ± 0.0010 [a]	0.0025 ± 0.0006 [a, b]
Mast cell density (cells per mm^2)	-2.13 ± 0.73	3.19 ± 0.74 [a]	2.55 ± 0.77 [a]

[a] Different from control group ($p<0.05$). [b] Different from unaffected side ($p<0.05$).

Supplementary Materials: figure e-1

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