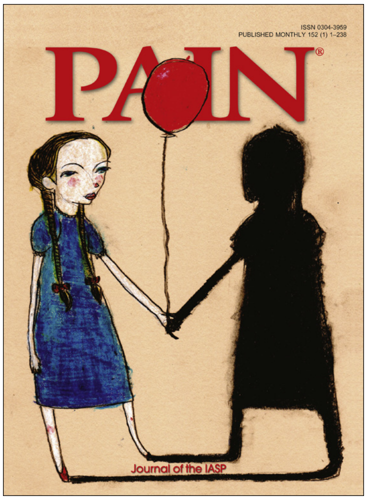

Up-regulation of α_1-adrenoceptors on cutaneous nerve fibres after partial sciatic nerve ligation and in complex regional pain syndrome type II

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Up-regulation of α_1-adrenoceptors on cutaneous nerve fibres after partial sciatic nerve ligation and in complex regional pain syndrome type II

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Introduction

After peripheral nerve injury, nociceptive afferents acquire an abnormal excitability to adrenergic agents that is linked with behavioural signs of pain [3; 11; 25; 26; 31; 34; 44]. Likewise, in chronic pain states such as post-herpetic neuralgia, cutaneous neuromas, amputation stump pain and complex regional pain syndrome (CRPS), intradermal administration of α_1-adrenoceptor (α_1-AR) agonists into affected sites may evoke aberrant pain that can persist for 30-60 minutes [2; 7-9; 27; 29; 51].

Within the central nervous system, quantitative radioligand binding studies in animal models have demonstrated an increased neural expression of α_1-ARs on surviving neurons after spinal transection or chemical lesion of adrenergic neurons [16; 37; 43]. Similarly, within the peripheral nervous system, messenger RNA for the α_{1B}-AR increased in rat dorsal root ganglia following peripheral nerve section or ligation of spinal nerves supplying those ganglia [31; 57]. Importantly, the proportion of dorsal root ganglion neurons that responded to norepinephrine increased markedly after nerve injury induced by loose or tight ligation of the sciatic nerve [39]. Conversely, the α_1-AR antagonist prazosin inhibited nociceptive fibre discharge in neuropathic pain models when given systemically [20; 23; 34]. Together, these findings suggest that an increased neural expression of α_1-ARs after peripheral nerve injury increases the excitability of nociceptive afferents.

We recently used immunohistochemistry to identify α_1-ARs on nociceptive fibres in normal rat skin [10], but whether α_1-AR expression changes on these nerve fibres after peripheral nerve injury is unknown. Hence, the aim of this study was to determine whether neural expression of α_1-ARs increased in the skin after partial sciatic nerve ligation (PSL) in rats, and in CRPS following a peripheral nerve injury in humans (causalgia or CRPS type II). PSL is a well-characterized model of CRPS type II associated with allodynia to light touch and hyperalgesia to noxious mechanical and thermal stimuli [45-47]. These pain behaviours are suppressed by intraperitoneal injection of guanethidine, an agent that displaces norepinephrine from sympathetic adrenergic neurons [30; 47]. Involvement of the sympathetic nervous system in CRPS is controversial [6]; nevertheless, adrenergic agonists aggravate pain in a subpopulation of CRPS patients [2; 9; 29; 51], and many patients with CRPS type II report long-term benefits after sympathectomy of the affected limb [5; 18]. Thus, we hypothesized that α_1-AR expression would increase on nerve fibres in the skin and sciatic nerve trunk after PSL in rats, and on nerve fibres in the skin of humans with CRPS type II.

Methods

Animals and surgery

Experiments were approved by the Murdoch University and Royal North Shore Hospital Animal Care and Ethics Committees. Tissues were obtained from 6 – 8 week old Wistar rats obtained from Animal Resources Centre (Canning Vale, Australia), following the guidelines of the 'NH&MRC Code of Practice for the Care and Use of Animals in Research in Australia'. Animals were housed in groups of three in individually ventilated cages under a 12-12 hour light-dark cycle, with environmental enrichment and free access to water and standard rat chow. The PSL model of nerve injury was performed using the method outlined by Seltzer et al. [45]. Briefly, the left sciatic nerve was exposed at mid-thigh level and a 4–0 silk suture inserted into the nerve to tightly ligate the dorsal 1/3 - 1/2 of the nerve trunk approximately 3 mm proximal to the trifurcation of the sciatic nerve [22]. The muscle (4–0) and then the skin (3–0) were closed with silk sutures. Sham-operated animals underwent the same surgery as the PSL-operated animals except that sutures were not inserted into the nerve and the nerve was not ligated. Six animals were examined in each experimental group. Mechanical allodynia was assessed prior to surgery (baseline) and again prior to perfusion either 4 or 28 days post-surgery in all animals using a series of Von Frey hairs to test the paw withdrawal threshold (range 0.2 g to 15 g) using the up-down paradigm outlined by Jayamanne et al. [22]. Animals were tested in batches of four mixed sham/PSL animals and the experimenter was blinded to the operation type.

Tissue processing and immunohistochemistry

Rats were heavily anesthetized with an intraperitoneal injection of sodium pentobarbitone (100mg/kg, Thiobarb, Jurox Pty. Ltd, NSW, Australia). Each rat was transcardially perfused with heparinized saline (0.2% v/v heparin, Mayne Pharma Pty Ltd, Melbourne, Australia and 0.9% w/v $NaCl_2$, pH 7.4) followed by 4% v/v formalin in 0.9% w/v $NaCl_2$, pH 7.3). The feet were then removed and postfixed in the 4% formalin solution for 4 hrs at room temperature. Plantar and hairy skin was collected from each hind paw, and then washed in 0.1M Phosphate Buffer (PB, pH 7.4) for 30 min, dehydrated in 50% v/v ethanol for 30 min and washed twice in PB for 15 min each. Tissues were cyroprotected by incubation for 48 hours in 30% w/v sucrose dissolved in tris phosphate buffered saline (TPBS, 0.1M PB, 0.9% w/v $NaCl_2$, 1% w/v tris base, pH 7.4) containing 0.1% w/v sodium azide (NaN_3) and then mounted in TissueTek® OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands) and stored at -80°C until use. 10 μm thick cryosections were cut using a cryostat (Leica CM 1850 UV, Nussloch, Germany) and collected onto silane coated slides (Hurst Scientific) and stored at -20°C until use.

Samples of the lesioned sciatic nerve were collected from four PSL animals four days after surgery and four sham operated animals at the same time point. Tissues were processed as described above.

For immunohistochemistry, sections were initially washed in 0.1M PBS (3x10 min) and incubated with 0.2% triton X-100 in PBS for 7.5 min at room temperature. Sections were washed with PBS (3x5 min) and blocked for 2 hrs in 10% donkey serum (Sigma) in PBS at room temperature. Sections were then treated with the following combinations of primary antibodies: α_1-AR/calcitonin gene related peptide (CGRP)/pan neuronal marker (TUJ1) to examine α_1-AR expression on peptidergic afferent nerve fibres; α_1-AR/ isolectin B4 (IB4)/TUJ1 to examine α_1-AR expression on non-peptidergic afferent nerve fibres; α_1-AR/ neurofilament 200 (NF200)/TUJ1 to examine α_1-AR expression on myelinated fibres; α_1-AR/ smooth muscle actin to examine α_1-AR expression on blood vessel walls; and myelin basic protein (MBP)/ NF200/α_1-AR to determine whether α_1-ARs were expressed on $NF200^+$ axons or co-localised with the myelin sheath surrounding these axons (details and dilutions are shown in Table 1). Antibodies were co-incubated for 48 hrs at 4^{o}C, diluted in blocking solution. Sections were then washed with PBS (3x15 min) and incubated with the appropriate species specific secondary antibodies (Table 1) diluted in 5% donkey serum (Sigma) in PBS for 4 hrs at room temperature. Sections were washed with PBS (3x15 min) and coverslipped with Prolong Gold anti-fade mounting media (Invitrogen). The pattern of staining produced by the α_1-AR antibody on blood vessels, nerves and keratinocytes resembles the staining pattern produced by BODIPY FL-prazosin, a fluorescent α_1-AR antagonist [10]. In addition, staining is eliminated following pre-adsorption of the anti-sera with an α_1-AR-specific peptide [10], and the peptide sequence recognized by the α_1-AR antibody is not found in any other G-protein-coupled receptor or non-receptor protein (National Center for Biotechnology Information, National Institutes of Health, BLAST program, http://blast.ncbi.nlm.nih.gov/Blast.cgi). A representative example of α_1-AR expression on the smooth muscle cells of large and small blood vessels is shown in supplementary Figure e1. Two consecutive sections per sample were stained and all immunohistochemistry for each staining combination was performed at the same time to ensure staining consistency. No staining was observed on negative control sections that had all primary antibodies omitted.

Human tissue processing and immunohistochemistry

Each participant provided written informed consent for the procedures, which were approved by the Human Research Ethics Committee at Murdoch University. Skin biopsies were collected from two women and three men aged between 23 and 56 years who met the clinical criteria for CRPS type II [32] (Table 2). The syndrome had begun 6 months to 10 years previously after accidental laceration of the ulnar nerve (two cases), surgery involving the sural or median nerve (two cases) or a crush injury of the sciatic nerve (one case). In each case, the injury was associated with a region of diminished sensation; however, pain and other sensory, autonomic and motor symptoms had spread beyond the distribution of the injured nerve. Pain decreased after sympathetic blockade in one patient but did not change in two other cases. Skin biopsies were collected under sterile conditions using a 3 mm diameter skin biopsy punch under local anaesthesia. Two biopsies were collected per patient; one from a region of hyperalgesia and another from the mirror image site on the contralateral limb. For comparison, skin samples were also collected from the dorsolateral aspect of one hand in four women and six men aged between 24 and 68 years (mean age 46 ± 15 years) without chronic pain.

The biopsies were fixed in Zamboni's solution (5% formalin and 1% picric acid in 0.9% saline) for 4 hours at 4°C, washed in PBS, incubated in 50% ethanol for 30 min followed by a further two rinses in PBS prior to paraffin embedding and sectioning into 10 μm sections that were collected onto silane coated slides (Hurst Scientific).

Sections were double-labelled for the α_1-AR and pan neuronal marker Protein Gene Product-9.5 (PGP9.5) using immunohistochemistry. As antibodies for each of these labels were raised in rabbit, the immunohistochemistry protocol was combined with tyramide signal amplification, which allows differentiation between two primary antibodies raised in the same species. Sections were deparaffinised through xylene and a descending series of ethanol washes to 0.1M PBS. Antigen retrieval was performed by incubation with 1mg/mL porcine trypsin (Sigma-Aldrich) for 20 min at 37°C. Sections were incubated with 3% H_2O_2 in PBS for 10 min to quench endogenous peroxidase activity, and then treated with 0.2% Triton X-100 in PBS for 5 min prior to incubation with blocking solution containing 10% normal donkey serum in PBS for 2 hrs. Next, sections were incubated with anti-PGP9.5 diluted 1:400,000 in blocking solution overnight at room temperature (rabbit anti-PGP9.5; AbD Serotec, USA, Product code 7863-0504), followed by biotin conjugated donkey anti-rabbit IgG for 1 hr (1:000; Jackson ImmunoResearch), streptavidin-horse radish peroxidase (1:150) for 30 min and biotin-conjugated tyramide (1:100 in amplification solution; Tyramide Signal Amplification Kit, NEL700; Perkin-Elmer Life Sciences) for 3 min. Sections were then incubated with DyLight 549-conjugated streptavidin (1:1000; Jackson ImmunoResearch) for 1 hr. Sequential double labelling was completed by incubation with rabbit anti-α_1-AR (1:250) diluted in blocking solution for

48hrs at 4°C followed by anti-rabbit DyLight-488 conjugated secondary antibody (1:600; Jackson ImmunoResearch) diluted in PBS for 4 hrs at room temperature. Sections were coverslipped with Prolong Gold.

Imaging and quantification of immunohistochemistry

Images of immunostained sections were collected using a Leica TCS SP2 multiphoton confocal microscope by an investigator who was blinded to side and group (PSL/sham and patient/control). Each label was collected sequentially at the appropriate excitation and emission spectra. For sciatic nerve sections, single images were acquired under 400x magnification, stacking a series of optical sections (Z-series) acquired at 1 μm intervals throughout the depth of the tissue. Skin sections were examined under 200X magnification with each z-series image collected every 2 μm. Two images, one of the papillary dermis containing the epidermis and the dermal nerve fibres, and one of the reticular dermis containing the large dermal nerve bundles, were collected for each skin section. Quantification was subsequently performed using the maximum projection image of the resulting stack. Care was taken to ensure that there was no bleed-through between channels using the imaging settings chosen, and all imaging settings were consistent for all sections in each staining run.

Both in rat and human skin samples, α_1-AR staining intensity was quantified in the epidermis, blood vessels, nerve fibres in the dermis and nerve fibres in large dermal nerve bundles by a blinded investigator. The epidermis was identified by morphology, blood vessels by smooth muscle actin, and nerve bundles and fibres in the dermis by pan-neuronal markers (TUJ1 for rat tissue and PGP9.5 for human tissue). In sections of rat sciatic nerve, α_1-AR staining intensity was quantified in axons, which were identified by TUJ1 staining. α_1-AR staining intensity in each region was quantified in at least two sections from each sample and averaged.

Quantification of α_1-AR staining intensity was performed using ImageJ software (Image J, version 1.47t, National Institute of Health, USA). To determine average pixel intensity on individual nerve fibres in the dermis or within nerve bundles, TUJ1 or PGP9.5 staining was used to create a mask of nerve fibre location, which was then applied to the corresponding α_1-AR stained image. This ensured that α_1-AR expression was only examined in those pixels also positive for TUJ1 or PGP9.5 staining. A similar approach was employed to quantify vascular α_1-ARs using smooth muscle actin to create a mask of blood vessel location. Quantification of α_1-AR intensity in the keratinocyte layer of the epidermis was performed by manually drawing around the keratinocyte layer and measuring the average α_1-AR intensity in the defined region.

α_1-AR staining intensity in rat tissue was also quantified in specific neuronal populations; peptidergic CGRP$^+$ neurons, non-peptidergic IB4$^+$ neurons, and myelinated NF200$^+$ neurons. For this quantification, a co-localisation analysis using the "Colocalization Finder" plugin for ImageJ was performed between each of these specific neuronal markers and TUJ1. This analysis allowed identification of all pixels that were immuno-positive for TUJ1 and individual neuronal markers, ensuring that all regions included in the resulting mask were these nerve fibres. This mask was then applied to the corresponding α_1-AR image and α_1-AR staining intensity was quantified in these neuronal populations.

Statistical approach

To combine data for neural markers across multiple immunohistochemistry runs, α_1-AR staining intensity within each run was expressed in normalized units for each region of interest (the keratinocyte layer of the epidermis, dermal blood vessels, nerve fibres in the papillary dermis, and nerve bundles in the reticular dermis). Raw scores from the ipsilateral and contralateral sides in lesioned and sham animals were combined into a single variable for each run and region, and transformed into scores with a mean of 0 and a standard deviation of 1 (i.e., Z-scores). Hence, positive scores represented greater than average α_1-AR staining intensity, whereas negative scores represented less than average α_1-AR staining intensity. These normalized scores were averaged across multiple immunohistochemistry runs for each animal to obtain a mean score for each region of interest. As the distribution and intensity of α_1-AR expression was similar in the plantar and hairy skin, these data were also averaged for quantification purposes. Mean scores were then compared between the injured and contralateral limb with Wilcoxon's matched-pairs signed-ranks tests, and between experimental and control groups with Mann-Whitney U tests. A similar approach was used to investigate differences in α_1-AR staining intensity in patients with CRPS Type II versus controls. Results are reported as the mean ± standard error, and the criterion of statistical significance was $p<0.05$.

Results

Mechanical allodynia after PSL

Prior to surgery all animals had a paw withdrawal threshold of 15 g. Paw withdrawal threshold in the ipsilateral limb was reduced to 0.4 ± 0.2 g at 4 days after PSL surgery and to 0.2 ± 0.1 g at 28 days after PSL. Paw withdrawal threshold was unchanged from baseline levels in all animals that underwent sham surgery.

α_1-AR expression in the skin after PSL

The strongest expression of α_1-ARs in the skin was observed in the epidermis. Strong α_1-AR expression was also observed in hair follicles, blood vessels and nerves, consistent with findings from our previous study [10]. This pattern of expression did not change in vascular or epidermal cells either 4 or 28 days after PSL.

PSL resulted in an up-regulation of α_1-ARs on papillary dermal nerve fibres identified by the pan-neuronal marker TUJ1. At 4 days after surgery, α_1-AR expression was significantly greater on nerve fibres in skin ipsilateral to injury than in animals that underwent sham surgery ($p = .015$) (Figure 1A). The intensity of α_1-AR expression in individual sensory nerve populations was then quantified to determine whether the up-regulation of α_1-AR expression was associated with specific nerve population(s). α_1-AR expression was significantly greater in non-peptidergic IB4$^+$ nerve fibres at 4 days post-PSL in comparison to animals that underwent sham surgery ($p = .032$), while α_1-AR expression was not significantly altered on peptidergic CGRP$^+$ nerve fibres or myelinated NF200$^+$

nerve fibres in the papillary dermis (Figure 1A). α_1-AR expression was no longer up-regulated on papillary dermal nerve fibres at 28 days after PSL (Figure 1B).

The intensity of α_1-AR expression was also quantified on axons in large nerve bundles in the deeper dermis. α_1-AR expression was significantly elevated in TUJ1$^+$ axons in skin ipsilateral to PSL at 4 days post-surgery in comparison to sham animals (p = .015) (Figure 1C). Specific, individual axons within nerve bundles were observed to have particularly high expression of α_1-AR after PSL (Figure 2), which was localized on axons and not in the surrounding connective tissue (Figure 2C). When α_1-AR expression in individual nerve populations was examined, it was found that α_1-AR staining intensity was significantly higher in IB4$^+$ axons at 4 days after surgery in skin ipsilateral to PSL in comparison to animals that underwent sham surgery (p = .017), although this was due largely to an outlier in the PSL group (Figure 1C). As shown in Figure 3, α_1-AR staining intensity within NF200$^+$ axons was also found to be significantly higher in skin ipsilateral than contralateral to PSL at 4 days after surgery (p = .028), and higher ipsilateral to PSL than in sham animals (p = .041) (Figure 1C). There was also a trend for higher α_1-AR expression in CGRP$^+$ axons after PSL in comparison to animals that underwent sham surgery, but with high variation among animals.

The up-regulation of α_1-AR expression on axons in larger nerve bundles persisted at 28 days after PSL. α_1-AR expression on TUJ1$^+$ axons remained significantly higher in skin ipsilateral to PSL in comparison to that from animals that underwent sham surgery (p = .009) (Figure 1D). α_1-AR staining intensity in skin ipsilateral to PSL remained significantly higher in NF200$^+$ axons in comparison to those from skin contralateral to PSL (p = .009) and from animals that underwent sham surgery (p = .028) (Figure 1D).

Co-localisation analysis for MBP, NF200 and α_1-AR revealed that the vast majority of α_1-AR staining co-localised with NF200, while there was minimal co-localisation with MBP (Figure 4). As NF200 stains intracellular neurofilaments in nerve fibres, this suggests that α_1-AR was up-regulated on axons and not in the surrounding myelin sheath.

α_1-AR expression in the sciatic nerve after PSL

α_1-AR was expressed on many axons within the sciatic nerve. Four days after PSL, α_1-AR expression further increased in IB4$^+$ neurons in comparison to surrounding axons that did not express IB4 (Figure 5), being 92 ±15% greater in IB4$^+$ neurons than in the non- IB4$^+$ surrounding axons 4 days after PSL surgery compared with 43 ±7% greater in IB4$^+$ neurons than in the surrounding non- IB4$^+$ axons 4 days after sham surgery (p = .043). This up-regulation was specific to IB4$^+$ axons as there were no significant differences in α_1-AR expression on axons in the sciatic nerve labelled with CGRP or NF200 between the PSL and sham groups.

α_1-AR expression in the skin of patients with CRPS type II

α_1-AR expression on nerve fibres in the dermis was greater in CRPS-affected limbs than in controls (p = .001) and, in each case, was greater in the CRPS-affected than unaffected limb of patients with CRPS type II (Figure 6A and Figure 7). Similarly, α_1-AR expression within the epidermis was greater in CRPS-affected limbs than in controls (p = .038) (Figure 6B and Figure 8). However, α_1-AR expression on blood vessels was similar in patients and controls. In the group as a whole, α_1-AR expression on dermal nerve fibres and keratinocytes did not differ significantly between the unaffected limb of patients with CRPS type II and controls (Figure 6).

Discussion

The aim of this study was to determine whether α_1-AR expression was up-regulated on nerve fibres in the skin in an animal model of CRPS type II and in patients with this condition. Four days after PSL, α_1-ARs were expressed more strongly on nerve fibres that survived the injury than on nerve fibres in animals that underwent sham surgery. This heightened α_1-AR expression was observed on non-peptidergic nociceptive afferents labelled with IB4 in the injured sciatic nerve, in dermal nerve bundles, and in the terminal distribution of these nerve fibres. Heightened expression of α_1-AR was also associated with NF200, a marker of myelinated nerve fibres, in dermal nerve bundles both 4 and 28 days after PSL. Co-localisation studies indicated that α_1-ARs were expressed on axons rather than on surrounding myelin or connective tissue. Furthermore, α_1-ARs were expressed more strongly on nerve fibres in affected than unaffected skin in patients with CRPS type II and also more strongly than that seen in the skin of pain-free controls. Together, these findings provide compelling evidence of an up-regulation of α_1-ARs on cutaneous nociceptive afferents after peripheral nerve injury. As each of the nerve fibre types that expressed α_1-ARs after nerve injury also express α_1-ARs in the uninjured state [10], this up-regulation appeared to involve heightened production of α_1-ARs rather than expression on nerve fibres that did not previously produce α_1-ARs.

Four days after sciatic nerve injury, α_1-ARs were up-regulated on IB4$^+$ nerve fibres. These non-peptidergic unmyelinated nerve fibres form a dense intra-epidermal network that protects the organism from potential harm by detecting noxious stimuli at the interface with the external environment [49]. This up-regulation was also detected in the sciatic nerve trunk, possibly due to increased axonal transport of α_1-ARs from production points in the soma or heightened membrane expression of α_1-ARs along the length of the unmyelinated nerve fibres.

Up to 28 days after nerve injury, up-regulation of α_1-AR expression was also observed on NF200$^+$ nerve fibres in dermal nerve bundles, but this did not extend to the sciatic nerve trunk or the papillary dermis. After peripheral nerve injury, NF200 is retained in only a small population of nerve fibres in the papillary dermis [38]. Thus, our findings suggest that α_1-AR expression was up-regulated on NF200$^+$ nerve fibres that lost NF200 in the papillary dermis, or that did not retain their superficial extensions after nerve injury. NF200 identifies medium- and large-diameter myelinated neurons, including A-delta nociceptors that express the tyrosine receptor kinase A (TrkA), a high-affinity receptor for nerve growth factor [4]. Many large-diameter nerve fibres that contain neurofilaments apparently lose their myelin before terminating in the skin [10; 41] and other peripheral tissues [19] (notably, most NF200$^+$ nerve fibres within the dermal nerve bundle shown in Figure 4B were unmyelinated). It would be interesting to determine whether α_1-ARs are expressed preferentially at nodal sites in myelinated axons, as this might explain why heightened co-localisation between α_1-AR and NF200 was limited to unmyelinated axons in dermal nerve bundles after PSL whereas α_1-AR expression was increased on unmyelinated IB4$^+$ fibres both in dermal nerve bundles and more proximally in the sciatic nerve.

Peripheral nerve injury evokes a complex response involving Wallerian degeneration, modifications in the phenotype of surviving neurons, neural regeneration and collateral sprouting. For example, a subpopulation of myelinated A-beta fibres that normally signal touch undergoes a phenotypic switch that involves increased expression of certain ion channels and membrane receptors [54], and an enhanced synthesis of nociceptive neurotransmitters such as substance P and CGRP [35]. This local response evokes changes in afferent signalling that result in central sensitisation, cortical remodelling and alterations in descending pain modulation processes [55; 58]. Wallerian degeneration is associated with an influx of immune cells into damaged nerves which, together with damaged axons, resident Schwann cells and target tissues, release inflammatory mediators and neurotrophins that reduce the firing threshold and induce transcriptional changes in production of neuropeptides, ion channels and membrane receptors in surviving neurons [14; 50; 55]. Neurotrophins such as nerve growth factor might trigger the synthesis of α_1-AR, as nerve growth factor augments the production of α_{1B}-AR in cultured dorsal root ganglion cells and increases their responsiveness to norepinephrine via actions on α_1-ARs [61]. In turn, heightened α_1-AR expression could augment neuroinflammatory responses by enhancing the release of inflammatory mediators such as substance P and prostaglandin E_2 [12; 24; 52]. Following partial peripheral nerve injury, sympathetic adrenergic fibres sprout in close proximity to nociceptive fibres, likely due to increased levels of nerve growth factor [28; 59]. The apposition of these two fibre types might permit aberrant crosstalk due to adrenergic excitation of up-regulated α_1-ARs on nociceptive fibres. α_1-AR activation generally increases neural excitability [17; 36; 40]; hence, stimulation of up-regulated α_1-ARs both on resident nociceptors and on myelinated afferents with *de novo* nociceptive properties could augment pain.

In a radioligand binding study, we previously found that α_1-AR density was greater in CRPS-affected skin than in the skin of normal controls [13], but the cellular source of this up-regulation could not be determined. The present findings suggest that cutaneous nerve fibres are an important source of heightened α_1-AR expression in the skin of patients with CRPS type II; however, the chief source might be keratinocytes, as α_1-AR expression was most intense in the epidermis, particularly on the affected side in patients with CRPS type II. The effect of this α_1-AR up-regulation on keratinocyte function is uncertain, but it is interesting that an elevated sodium channel expression in the keratinocytes of patients with CRPS type I may increase their excitability [62]. Keratinocytes are a major source of cutaneous nerve growth factor which, after peripheral nerve injury, can stimulate hyperexcitability in regenerating afferent nerve fibres [42]. Thus, in the presence of adrenergic agonists such as epinephrine or norepinephrine, liberation of inflammatory mediators or growth factors from hyperexcitable keratinocytes might contribute to pain. However, this speculation will have to be confirmed in further studies.

In contrast to patients with CRPS type II, α_1-AR expression was not altered on keratinocytes in the injured limb of rats after PSL. The basis of this discrepancy is unclear, but might involve differences in timing (4-28 days after injury in rats compared with months or years after peripheral nerve injury in patients with CRPS type II), variation in the location or extent of injury between the animal model and the human condition, or differences in the intensity of the neuroinflammatory response that might trigger α_1-AR up-regulation. In addition, heightened expression of α_1-ARs on dermal nerve fibres was detected in CRPS patients many months after peripheral nerve injury, but had resolved in the papillary dermis within 28 days of PSL in the rodent model. Whether this represents a species difference or a characteristic of CRPS is unknown.

Vascular sensitivity to vasoconstrictor agents, including α_1-AR agonists, increases after nerve injury [53; 60]. Nevertheless, in the present study, vascular expression of α_1-ARs did not change after PSL, and was similar in patients with CRPS type II and controls. Loss of neuronal noradrenaline transporters or other changes (e.g., in gap-junction proteins, angiotensin II receptor expression or intracellular signalling to contractile stimuli) may contribute to denervation supersensitivity [53]. Alterations in circulatory dynamics resulting from this nonspecific increase in vascular sensitivity could augment inflammation and/or pain [56].

Overall, our results indicate that α_1-AR expression increases on keratinocytes and nociceptive afferents after peripheral nerve injury both in a well-characterized animal model of CRPS type II and in the human condition. Only one of three patients in this small series clearly benefited from regional sympathetic blockade, and none had been treated with adrenergic blocking agents. Nevertheless, in uncontrolled trials, non-specific α-adrenergic antagonists such as phentolamine and phenoxybenzamine [15; 21; 33] and specific α_1-AR antagonists such as prazosin and terazosin decreased the pain of CRPS [1; 48]. The present findings establish a mechanistic basis for this therapeutic effect in patients with CRPS type II. In particular, α_1-antagonists may block the actions of circulating or locally secreted catecholamines on neural α_1-ARs, or prevent the adrenergic activation of keratinocytes. Thus, further exploration of the therapeutic potential of α_1-blockers for CRPS type II seems warranted.

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Conflicts of interest statement

None of the authors has a conflict of interest with the contents of this paper.

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Figure legends

Supplementary Figure e1: α_1-AR expression (red) on the smooth muscle cells (green) of a large artery in cross-section and a small blood vessel cut longitudinally (upper left). Scale bar = 50 µm.

Figure 1: Quantification of α_1-AR staining intensity in normalised units (Z scores) on nerve fibres in the papillary dermis and in large dermal nerve bundles at 4 (A,C) and 28 days (B,D) after PSL. α_1-AR staining intensity was quantified in 4 different neuronal populations: all neurons labelled with the pan-neuronal marker TUJ1; peptidergic neurons labelled with CGRP; non-peptidergic neurons labelled with IB4; and myelinated neurons labelled with NF200. * indicates significant differences between ipsilateral (filled circles) and contralateral skin (open circles) and # indicates significant differences between PSL and sham animals ($p<0.05$). The horizontal bar in each column represents the median.

Figure 2: α_1-AR expression (red) was up-regulated in dermal nerve bundles in skin ipsilateral to PSL (A) in comparison to skin contralateral to PSL (D) and that from animals that underwent sham surgery (G). Representative images at 4 days after PSL or sham surgery are shown. Nerve fibres were identified using the pan-neuronal marker TUJ1 (blue; B, E, H). Co-localized images highlight all pixels expressing TUJ1 and a high concentration of α_1-AR in white. Scale bar = 50 µm

Figure 3: Strong α_1-AR (red) up-regulation was observed in NF200 labelled fibres (green) in nerve bundles after PSL (A-C) in comparison to those in skin contralateral to PSL (D-F) and after sham surgery (G-I). Representative images at 4 days after PSL or sham surgery are shown. Co-localized images (C, F, I) highlight all pixels that expressed NF200 and a high concentration of α_1-AR in white. Scale bar = 50 µm

Figure 4: Nerve bundle from a skin section at 4 days after PSL stained with triple immunohistochemistry for α_1-AR (red), myelin basic protein (MBP; green) and NF200 (blue). Specific axons expressed high levels of α_1-AR (A). B shows a merged image of all markers. Co-localization

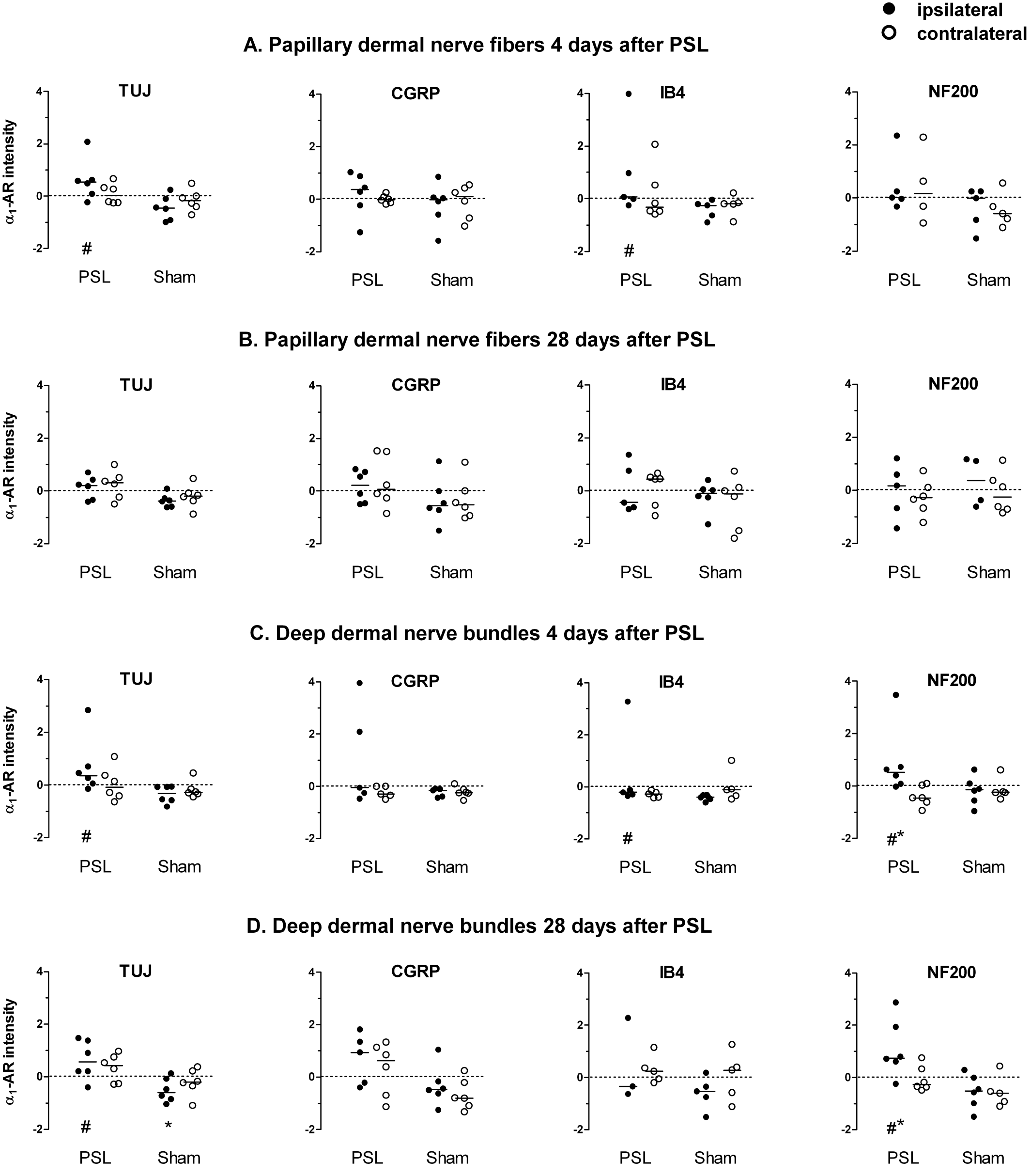
analysis was then performed identifying pixels containing co-localized MBP and α_1-AR (C) and NF200 and α_1-AR (D). All pixels containing co-localized markers are shown in white in both C and D. Note the minimal co-localisation of α_1-AR with MBP in B and C. Scale bar = 20 μm.

Figure 5: α_1-AR expression was up-regulated in IB4 axons in the sciatic nerve at 4 days after PSL (A-D) in comparison to sciatic nerve after sham surgery (E-H). Arrows show axons that are co-labelled with α_1-AR, IB4 and TUJ1, which also have up-regulated expression of α_1-AR. Scale bar = 50 μm

Figure 6: Quantification of α_1-AR staining intensity in normalised units (Z scores) on (A) dermal nerve fibres and (B) in the epidermis of patients with CRPS type II and controls. # α_1-AR expression was greater in the CRPS-affected limb than in controls ($p<0.05$). The horizontal bar in each column represents the median.

Figure 7: α_1-AR expression (red) was up-regulated on dermal nerve fibres (labelled green) in CRPS-affected skin in comparison to nerve fibres from the same patient in unaffected skin and in a representative pain-free control. Nerve fibres were identified using the pan-neuronal marker PGP9.5. Co-localized images highlight all pixels that expressed PGP9.5 and a high concentration of α_1-AR in white. α_1-AR was also expressed in the perineurium surrounding the nerve fibres (red in the co-localized images). Scale bar = 20 μm in the columns illustrating α_1-AR and PGP-9.5 staining of dermal nerve fibres, and 50 μm in images illustrating α_1-AR (red) and PGP-9.5 staining (green) more broadly in the epidermis and dermis. The boxed areas in images in the far-right column show the location of nerve fibres illustrated at higher magnification in the accompanying images.

Figure 8: α_1-AR expression (red) was up-regulated in the epidermis in CRPS-affected skin in comparison to pain-free controls. Scale bar = 50 μm

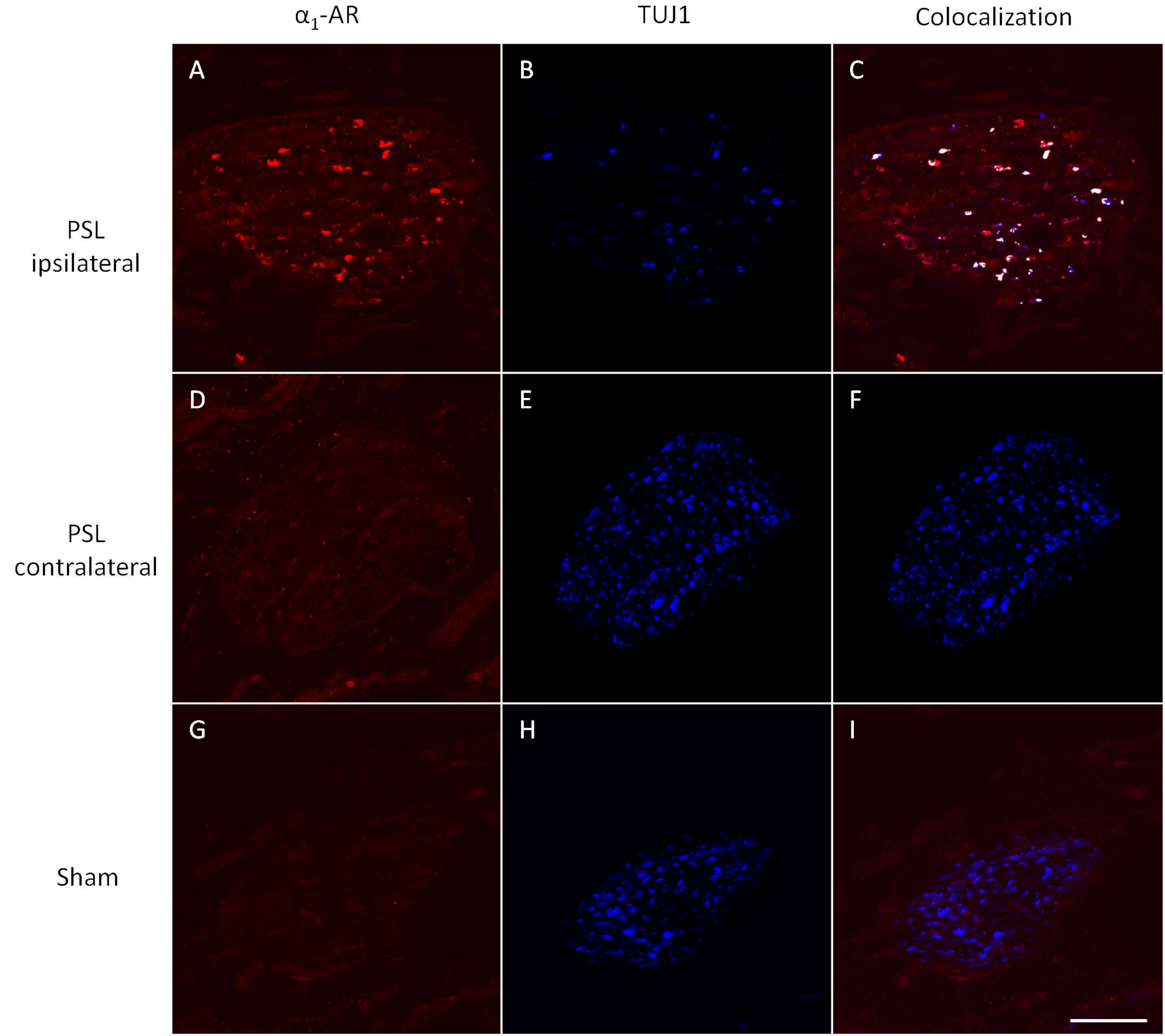


A. Papillary dermal nerve fibers 4 days after PSL

B. Papillary dermal nerve fibers 28 days after PSL

C. Deep dermal nerve bundles 4 days after PSL

D. Deep dermal nerve bundles 28 days after PSL


α_1-AR
TUJ1
Colocalization
PSL ipsilateral
PSL contralateral
Sham
A
B
C
D
E
F
G
H
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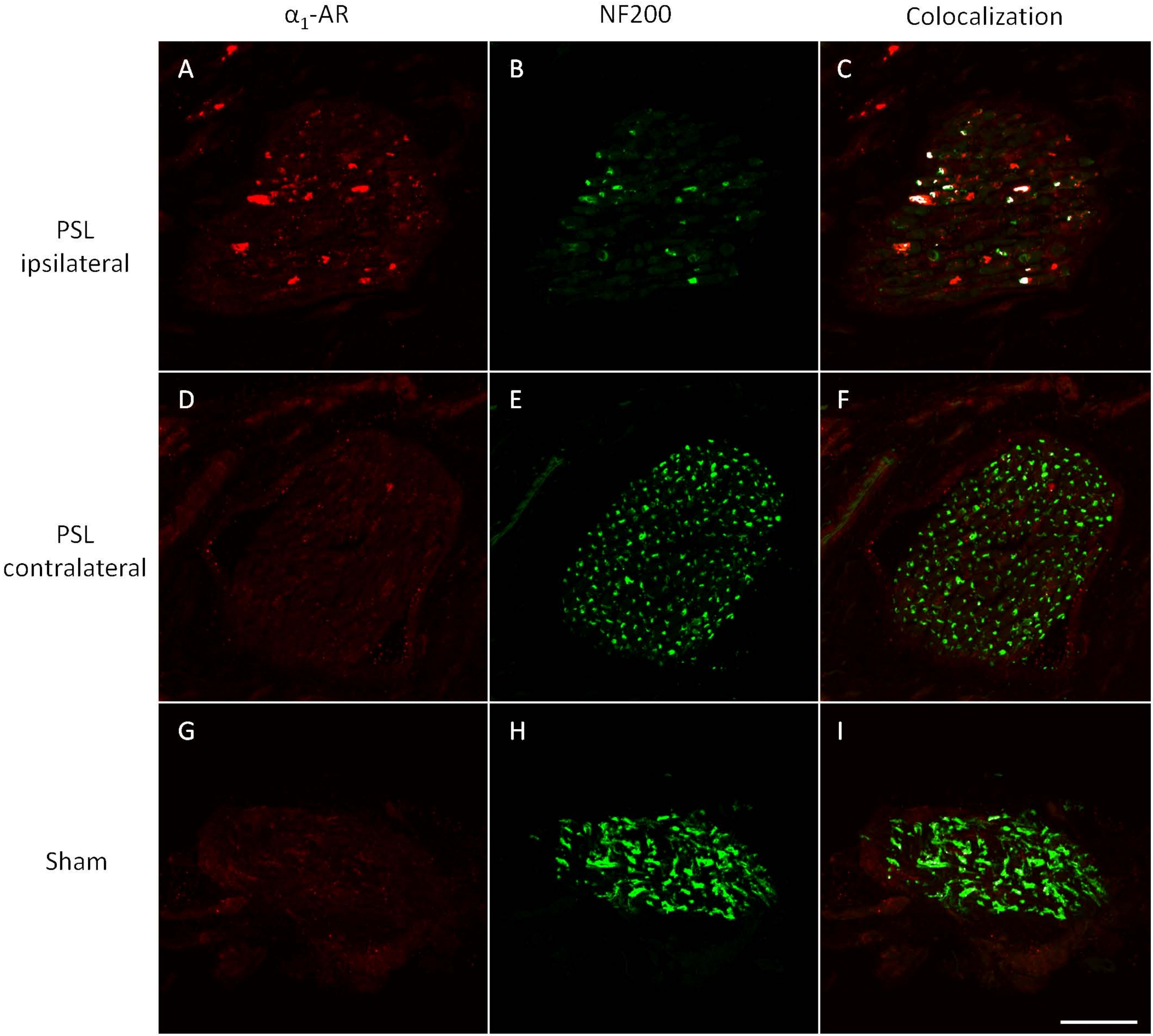
α_1-AR
NF200
Colocalization
PSL ipsilateral
PSL contralateral
Sham
A
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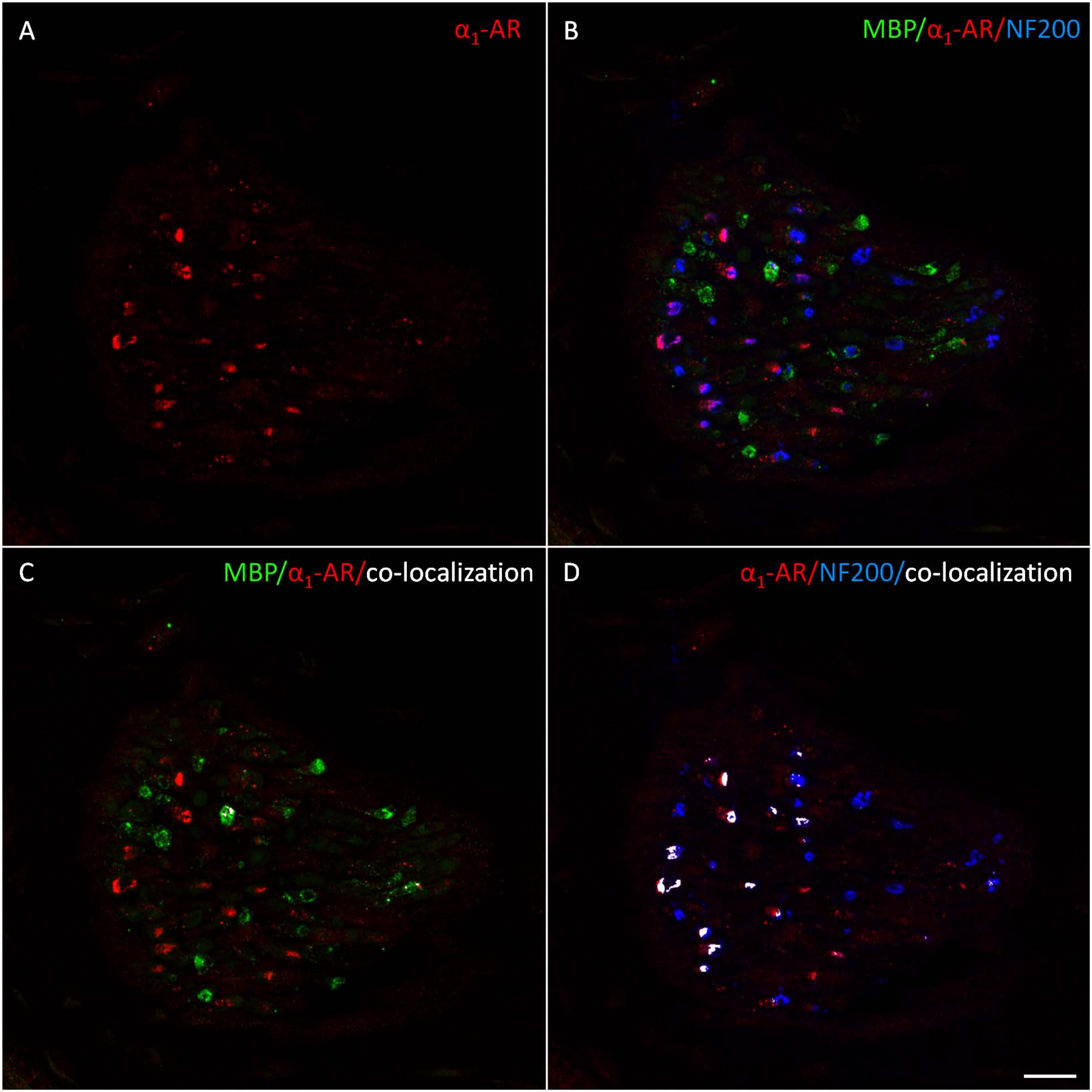
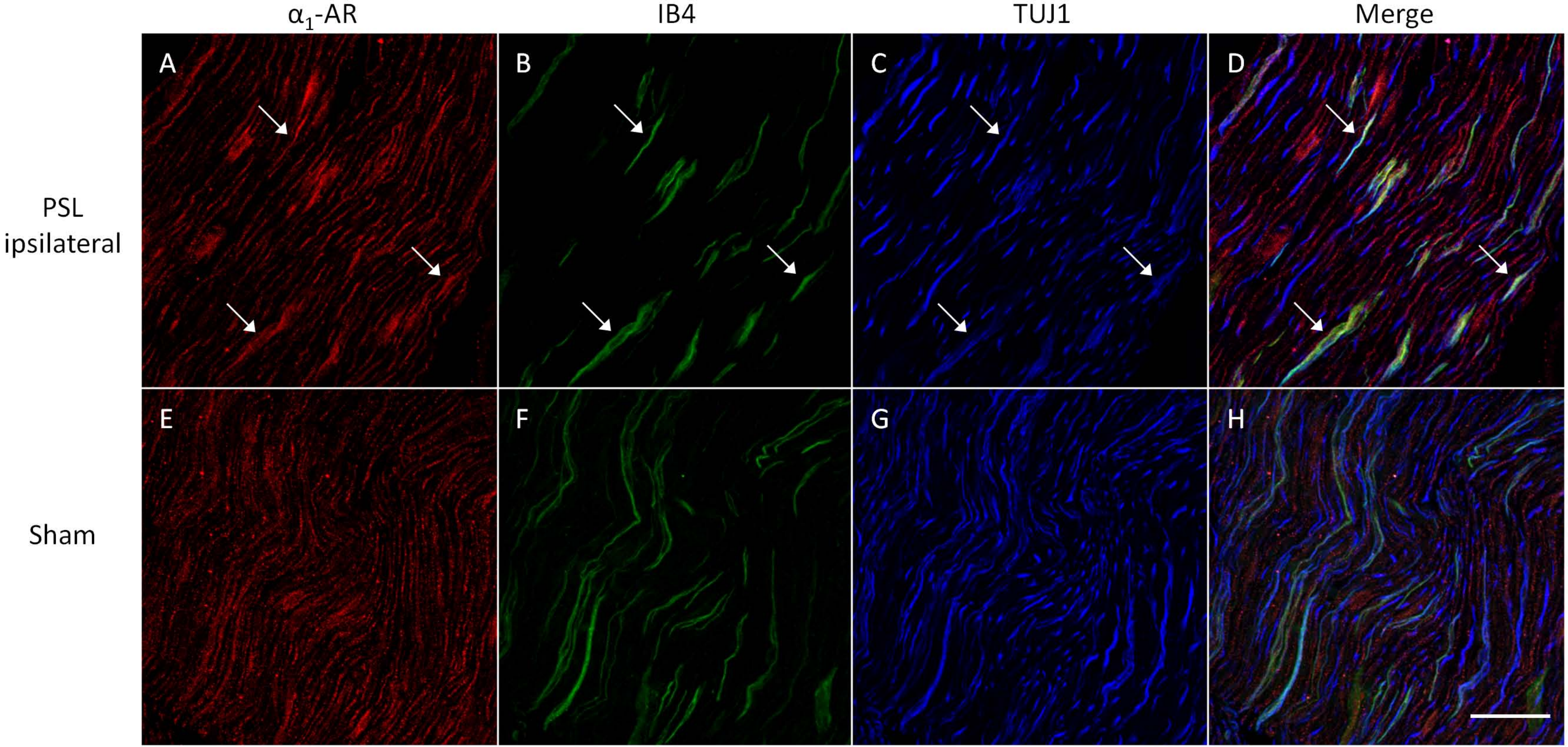
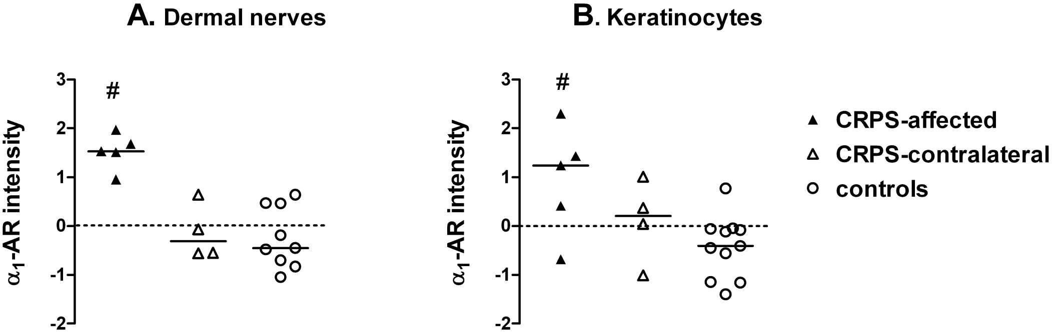
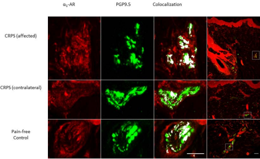

A
α_1-AR
B
MBP/α_1-AR/NF200
C
MBP/α_1-AR/co-localization
D
α_1-AR/NF200/co-localization


α_1-AR
IB4
TUJ1
Merge
PSL ipsilateral
Sham
A
B
C
D
E
F
G
H


A. Dermal nerves
B. Keratinocytes
α_1-AR intensity
3
2
1
0
-1
-2
#
▲ CRPS-affected
△ CRPS-contralateral
○ controls


α_1-AR
PGP9.5
Colocalization
CRPS (affected)
CRPS (contralateral)
Pain-free Control

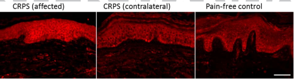

CRPS (affected)
CRPS (contralateral)
Pain-free control

Table 1. Primary and secondary antibodies used in rat tissues: dilutions and source

Antigen and host species	**Dilution - skin**	**Dilution – sciatic nerve**	**Product code and source**
isolectin B4, FITC conjugate	1:250	1:250	L2895, Sigma-Aldrich
Primary antibodies			
anti α_1-AR, rabbit polyclonal	1:200	1:250	A270, Sigma-Aldrich
anti neuronal class III β-tubulin (TUJ1), mouse monoclonal	1:800	1:2000	MMS-435P, Covance
anti CGRP, goat polyclonal	1:400	1:2000	1720-9007, AbD Serotec
anti NF200, chicken polyclonal	1:4000	1:7500	Jackson ImmunoResearch
anti MBP, mouse monoclonal	1:400	n/a	Sc-66064, Santa Cruz
anti SMA, mouse monoclonal	1:4000	n/a	A2547, Sigma-Aldrich
Secondary antibodies			
Donkey anti-chicken Cy2	1:600	1:600	Jackson ImmunoResearch
Donkey anti-goat DyLight-488	1:600	1:600	Jackson ImmunoResearch
Donkey anti-rabbit Dylight-549	1:1200	1:1200	Jackson ImmunoResearch
Donkey anti-mouse Dylight-647	1:1000	1:1000	Jackson ImmunoResearch

Abbreviations: α_1-adrenoceptor (α_1-AR), Calcitonin Gene Related Peptide (CGRP), Myelin Basic Protein (MBP), Smooth Muscle Actin (SMA), Neurofilament 200 (NF200), Fluorescein Isothiocyanate (FITC), Cyanine (Cy2).

Table 2. Clinical characteristics of patients with CRPS type II

Age, sex	Duration	Injury	Symptoms noted during the physical examination	Effect of regional sympathetic blockade
56, F	10 years	sciatic nerve crush	Allodynia, very swollen, very cold, cyanotic, decreased range of motion	Pain decreased
48, M	23 months	ulnar nerve laceration	Allodynia, mildly swollen, cold, decreased range of motion, mild dystonia, tremor	Not tried
45, F	46 months	carpal tunnel surgery	Allodynia, mildly swollen, warm, increased sweating, decreased range of motion, severe dystonia, tremor	Severe nausea
44, M	6 months	fibula fracture, sural nerve surgery	Allodynia, mildly swollen, cyanotic, decreased range of motion	Not tried
23, M	6 months	ulnar nerve laceration	Allodynia, mildly swollen, cyanotic, warm, decreased range of motion	No change in pain

Abstract

After peripheral nerve injury, nociceptive afferents acquire an abnormal excitability to adrenergic agents, possibly due to an enhanced expression of α_1-adrenoceptors (α_1-AR) on these nerve fibres. To investigate this in the present study, changes in α_1-AR expression on nerve fibres in the skin and sciatic nerve trunk were assessed using immunohistochemistry in an animal model of neuropathic pain involving partial ligation of the sciatic nerve. In addition, α_1-AR expression on nerve fibres was examined in painful and unaffected skin of patients who developed complex regional pain syndrome after a peripheral nerve injury (CRPS type II). Four days after partial ligation of the sciatic nerve, α_1-AR expression was greater on dermal nerve fibres that survived the injury than on dermal nerve fibres after sham surgery. This heightened α_1-AR expression was observed on non-peptidergic nociceptive afferents in the injured sciatic nerve, dermal nerve bundles and the papillary dermis. Heightened expression of α_1-AR in dermal nerve bundles after peripheral nerve injury also co-localised with neurofilament 200, a marker of myelinated nerve fibres. In each patient examined, α_1-AR expression was greater on nerve fibres in skin affected by CRPS than in unaffected skin from the same patient or from pain-free controls. Together, these findings provide compelling evidence of an up-regulation of α_1-ARs on cutaneous nociceptive afferents after peripheral nerve injury. Activation of these receptors by circulating or locally secreted catecholamines might contribute to chronic pain in CRPS type II.