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Increased expression of cutaneous α_1-adrenoceptors after chronic constriction injury in rats

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Abstract

Alpha$_1$-adrenoceptor expression on nociceptors may play an important role in sympathetic-sensory coupling in certain neuropathic pain syndromes. The aim of this study was to determine whether α_1-adrenoceptor expression was up-regulated on surviving peptidergic, non-peptidergic and myelinated nerve fiber populations in the skin after chronic constriction injury of the sciatic nerve in rats. Seven days after surgery, α_1-adrenoceptor expression was up-regulated in the epidermis and on dermal nerve fibres in plantar skin ipsilateral to the injury, but not around blood vessels. This α_1-adrenoceptor up-regulation in the plantar skin was observed on all nerve fiber populations examined. However, α_1-adrenoceptor expression was unaltered in dorsal hind paw skin after the injury. The increased expression of α_1-adrenoceptors on cutaneous nociceptors in plantar skin after chronic constriction injury suggests that this may be a site of sensory-sympathetic coupling that increases sensitivity to adrenergic agonists after nerve injury. In addition, activation of up-regulated α_1-adrenoceptors in the epidermis might cause release of factors that stimulate nociceptive signalling.

Perspective: Our findings indicate that peripheral nerve injury provokes up-regulation of α_1-adrenoceptors on surviving nociceptive afferents and epidermal cells in the skin. This might contribute to sympathetically maintained pain in conditions such as complex regional pain syndrome, painful diabetic neuropathy and post-herpetic neuralgia.

Keywords: α_1-adrenoceptors; nociceptors; neuropathic pain; chronic constriction injury; keratinocytes; sensory-sympathetic coupling

Introduction

Activation of the sympathetic nervous system with consequent release of norepinephrine does not normally activate nociceptors. However, after injury, sensory-sympathetic coupling in the skin may become a source of pain by activating α_1-adrenoceptors (α_1-ARs) on nociceptive afferent fibers [1, 10, 12, 28, 43]. After nerve injury, messenger RNA for the α_{1B}-AR subtype increases in the dorsal root ganglia [29, 50], but whether this results in an up-regulation of α_1-ARs on nociceptors in the skin is unknown.

Chronic constriction injury (CCI) of the rat sciatic nerve triggers signs of pain and hyperalgesia as early as 24 hours post-injury [3, 5, 9, 18], together with neurodegeneration of sensory nerve fibers in the injured sciatic nerve and the skin. The injured nerve fibers regenerate gradually in the weeks following injury, resulting in a *de novo,* spatially intimate association of sprouting sympathetic efferent fibers and sensory afferent fibers in the upper dermis [25-27, 37, 51]. Chemical and surgical sympathectomy generally attenuates allodynia and thermal hyperalgesia after CCI [7, 34] (but see [18]), suggesting that certain features of the neuropathic pain associated with CCI may be sympathetically maintained. Therefore, the aim of this study was to determine whether α_1-AR expression was up-regulated on surviving peptidergic, non-peptidergic and myelinated nerve fiber populations in the skin after CCI. As α_1-AR expression in the epidermis and on blood vessels could indirectly influence nociceptor signalling, α_1-AR expression changes on these skin structures were also examined.

Methods

Chronic constriction injury

The experiments conformed to the ethical guidelines of the International Association for the Study of Pain, the National Institutes of Health (USA), and the Canadian Institutes of Health Research. All protocols were approved by the Animal Care Committee of the Faculty of Medicine, McGill University, in accordance with the regulations of the Canadian Council on Animal Care, and by the Animal Ethics Committee of Murdoch University. All animals used in this study were male Sprague

Dawley rats weighing 175-200g. Six rats underwent unilateral CCI of the common sciatic nerve [5] and three rats were examined as naive, un-operated age-matched controls.

Histological processing and immunohistochemsitry

Seven days after surgery rats were anesthetised with a lethal dose of sodium pentobarbital (100 mg/kg, IP) and perfused transcardially with a vascular rinse (0.1M phosphate buffered saline containing 0.05% sodium bicarbonate and 0.1% sodium nitrite) followed by freshly prepared 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4. The hind paws were severed and post-fixed overnight, after which a sample of glabrous, plantar skin was excised from the wide part of the plantar hind paw that lies distal to the calcaneus and proximal to the digital tori and another sample of skin was taken from the dorsum of the hind paw proximal to the toes. The samples were cryoprotected in 30% sucrose at 4°C overnight and then embedded in Optimal Cutting Temperature compound, frozen on dry ice, and stored at -80°C. 10 μm thick cross-sections were cut using a cryostat and collected onto silane coated slides (Hurst Scientific).

Cryosections were stained with the following combinations of antibodies (details shown in Table 1): α_1-AR/calcitonin gene related peptide (CGRP)/pan-neuronal marker (TUJ1) to examine α_1-AR expression on peptidergic afferents; α_1-AR/isolectin B4 (IB4)/TUJ1 to examine α_1-AR expression on non-peptidergic afferents; α_1-AR/neurofilament 200 (NF200)/TUJ1 to examine α_1-AR expression on myelinated fibers; and α_1-AR/smooth muscle actin (SMA) to examine α_1-AR expression on blood vessels.

For immunohistochemistry, sections initially were washed in 0.1M PBS (3x10 min) and then incubated with 0.2% Triton X-100 for 7.5 min at room temperature. Sections were washed with PBS (3x5 min) and blocked for 2 hrs in 10% donkey serum in PBS at room temperature. Sections were incubated for 48 hrs at 4°C with primary antibodies using the concentrations shown in Table 1, diluted in blocking solution. Sections were washed with PBS (3x15 min) and then incubated with the

appropriate secondary antibodies diluted in 5% donkey serum (Sigma) in PBS for 4 hrs at room temperature. Sections were washed with PBS (3x15 min) and cover-slipped with Prolong Gold anti-fade mounting media.

The peptide sequence recognized by the α_1-AR antibody used in this study is unique to the α_1-AR, a G-protein-coupled receptor (National Center for Biotechnology Information, National Institutes of Health, BLAST program, http://blast.ncbi.nlm.nih.gov/Blast.cgi). In an examination of the specificity of the α_1-AR antibody, the pattern of staining on blood vessels, nerves and epidermal cells in rat skin tissue resembled the staining pattern produced by BODIPY FL-prazosin, a fluorescent α_1-AR antagonist. [6] In addition, staining was eliminated following pre-adsorption of the anti-sera with an α_1-AR-specific peptide. [6] In the present study, no staining was observed on negative control sections that had the primary antibodies omitted. Two consecutive sections per sample were stained with a combination of antibodies, and immunohistochemistry for each antibody combination was performed in one run to ensure staining consistency.

Quantification of Immunohistochemistry

α_1-AR staining intensity was quantified in the epidermis, dermal blood vessels, nerve fibers in the upper dermis, and nerve fibers in large dermal nerve bundles by a blinded investigator. The epidermis was identified by morphology, blood vessels were identified by SMA staining and nerve fibers in the dermis and nerve bundles were identified using the pan-neuronal marker TUJ1. α_1-AR staining intensity in the epidermis and nerve fibers was quantified in 7 sections per sample and averaged. Since many blood vessels were present in each skin section, α_1-AR staining intensity in small blood vessels in the papillary dermis proximal to the epidermis was quantified as the average intensity in the smooth muscle surrounding all blood vessels in two representative images taken from one section per sample. One additional image from each α_1-AR/SMA stained section was

collected from the deeper reticular dermis and used to quantify α_1-AR staining intensity in the smooth muscle surrounding large blood vessels.

Images of immunostained skin sections were collected using a Nikon A1 confocal microscope. For quantification of α_1-AR intensity, two 200X magnification confocal image stacks were collected per section; one image stack contained the papillary dermis, epidermis and dermal nerve fibers, and the other image stack was of the large dermal nerve bundles in the reticular dermis. Two 200X images of the papillary dermis and one from the reticular dermis were collected from sections stained with α_1-AR and SMA for quantification of α_1-AR expression on blood vessels. All confocal stacks consisted of consecutive images collected with an optical section thickness of 2 μm, and the maximum intensity projection overlay of the resulting stack was used for quantification. Special care was taken to ensure that there was no bleed-through between channels using the imaging settings chosen, and imaging settings were identical for all sections in each staining run.

Quantification of immunohistochemistry staining intensity was performed using ImageJ software (available from http://rsbweb.nih.gov.ij). The proportion of each nerve bundle occupied by nerve fibers was calculated in 4-7 nerve bundles per sample. Nerve bundles were identified using a combination of morphology and TUJ1 staining. The perimeter of each nerve bundle was manually traced around and the area was measured. An intensity threshold was applied to all TUJ1 images, which ensured that only immuno-positive TUJ1 ($TUJ1^+$) pixels were included in analysis, and the proportion of $TUJ1^+$ pixels in each nerve bundle was determined.

α_1-AR staining intensity was measured as the average pixel intensity in each area of interest.TUJ1 staining was used to identify the location of nerve fibers, which was then used as a mask on the corresponding α_1-AR stained image, ensuring that α_1-AR expression was only examined in those pixels also positive for TUJ1 staining. Similarly, quantification of the average α_1-AR intensity on blood vessels was performed by creating a mask from SMA immunostaining and using that to define the

location of blood vessels in the corresponding α_1-AR image. Quantification of α_1-ARs in 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 was also examined in specific primary afferent neuron subpopulations: peptidergic CGRP$^+$ neurons, non-peptidergic IB4$^+$ neurons and myelinated NF200$^+$ neurons. For this quantification, a co-localisation analysis was performed between each of these specific neuronal markers and TUJ1 using the "Co-localization Finder" plugin to create a mask of pixels that identified each individual neuronal population. This mask was applied to the corresponding α_1-AR image and α_1-AR staining intensity was then quantified in these specific neuronal populations. The proportion of nerve fibers belonging to each of these populations was quantified by expressing the number of pixels co-localized for TUJ1 and CGRP, IB4 or NF200 as a percentage of the total number of pixels labelled for TUJ1.

Statistical approach

To combine data for neural markers across multiple immunohistochemistry runs, α_1-AR$^+$ pixel intensity within each run was expressed as a Z-score for each region of interest (nerve fibers in the papillary dermis and nerve bundles in the reticular dermis). Mean scores were then compared between the injured and contralateral limb with Wilcoxon's Signed Ranks test, and between experimental and control animals with the Mann-Whitney U test. A similar approach was used to investigate differences in α_1-AR$^+$ pixel intensity in the epidermis and blood vessel walls, and in peptidergic, non-peptidergic and myelinated neuron populations throughout the papillary and reticular dermis. Results are reported as the mean ± standard error, and the criterion of statistical significance was $p<0.05$.

Results

Neurodegeneration after CCI

The proportion of pixels within nerve bundles labelled by the pan-neuronal marker TUJ1 was used to index the extent of neurodegeneration. There was a trend for this index to be lower in skin ipsilateral than contralateral to CCI and in comparison to skin from naive animals both in plantar and hairy skin (Table 2), but this trend did not achieve statistical significance.

The surviving dermal $TUJ1^+$ fibers were then separated into peptidergic, non-peptidergic and myelinated neuron populations, and the proportions of these populations were compared across experimental groups to determine whether any of these populations were particularly vulnerable to CCI. There was a profound degeneration of $NF200^+$ fibers in plantar skin ipsilateral to CCI (Figure 1); the percentage of $NF200^+$ pixels co-labelled for TUJ1 decreased to 6±2% of the total number of $TUJ1^+$ pixels, which was lower than in skin contralateral to CCI (24±5%, $p<0.05$) and in naive animals (28± 6%, $p<0.05$). This degeneration was specific to $NF200^+$ fibers as the proportions of $CGRP^+$ and $IB4^+$ pixels remained unchanged after CCI (Table 3). Interestingly, the degeneration of myelinated fibers was observed in plantar skin but not in hairy skin on the dorsal paw (Figure 1 and Table 3).

α_1-AR expression in skin of naïve rats

The strongest α_1-AR expression was observed in the epidermis. α_1-AR expression was also observed around blood vessels and in nerve fibers in the dermis and in large dermal nerve bundles. The strong α_1-AR expression throughout the epidermis precluded observation of α_1-ARs on intra-epidermal nerve fibers. The pattern of α_1-AR staining was consistent with a previous study that examined α_1-AR staining in skin from uninjured rats [6].

α_1-AR up-regulation in plantar skin after nerve injury

α_1-AR expression was up-regulated after CCI in all regions examined in ipsilateral plantar skin except around blood vessels. CCI resulted in significantly increased α_1-AR expression on nerve fibers labelled with TUJ1 ($TUJ1^+$) and in nerve bundles in the deep dermis in comparison to skin contralateral to CCI ($p<0.05$) and to skin from naive animals ($p<0.05$) (Figure 2, Figure 3A). α_1-AR expression was also significantly higher in $TUJ1^+$ nerve fibers in the papillary dermis in skin ipsilateral to CCI than in contralateral skin ($p<0.05$) (Figure 3B).

α_1-AR expression was then examined in individual primary afferent subpopulations. In those nerve fibers that remained after injury, α_1-AR expression was significantly higher on $CGRP^+$ fibers in skin ipsilateral than contralateral to CCI ($p<0.05$) (Figure 4A). α_1-AR expression was also significantly higher in $IB4^+$ fibers in skin ipsilateral than contralateral to CCI ($p<0.05$) and in comparison to skin from naive animals ($p<0.05$) (Figure 4B). Similarly, in the few remaining plantar dermal $NF200^+$ fibers, α_1-AR expression was higher ipsilateral than contralateral to CCI ($p<0.05$) (Figure 4C).

α_1-AR expression was significantly higher in the epidermis ipsilateral than contralateral to CCI ($p<0.05$) and in comparison to skin from naive animals ($p<0.01$) (Figure 3C, Figure 5). There was no difference in α_1-AR expression in the epidermis from skin contralateral to CCI compared to skin from naive animals. There were also no significant differences in the average α_1-AR staining intensity in small blood vessels in the papillary dermis or large blood vessels in the reticular dermis after CCI in comparison to skin contralateral to CCI or to skin from naive animals (Figure 6A).

α_1-AR expression in hairy skin

α_1-AR expression was not altered in the epidermis, dermal blood vessels, nerve fibers in the dermis or nerve fibers in dermal nerve bundles in hairy skin after CCI in comparison either to skin from the contralateral paw or from naive rats (Figure 3D-F and Figure 6B). As immunohistochemistry of hairy skin was performed at the same time as plantar skin, and α_1-AR staining intensity was consistent

between groups in all structures examined in hairy skin, these results suggest that α_1-AR expression was not up-regulated in hairy skin at 7 days post-CCI.

Discussion

α_1-AR expression was increased on cutaneous nerve fibers and in the epidermis after CCI. We have previously shown that nociceptors express α_1-ARs basally [6]. Others have shown that surviving nerve fibers become more sensitive to α_1-AR agonists after nerve injury [2, 33] and that administration of α_1-AR antagonists reduces thermal and mechanical hyperalgesia in animal models of neuropathic pain [14, 19, 20, 22, 49]. The increased expression of α_1-ARs on cutaneous nociceptors after CCI suggests that this may be a site of sensory-sympathetic coupling, and could provide a mechanism underlying this hypersensitivity to adrenergic agonists after nerve injury. It has been hypothesised that direct activation of α_1-ARs on non-peptidergic nociceptors expressing the P2X3 receptor enhances the firing rate to painful stimuli by activating protein kinase C [29, 31]. Considering that we also found α_1-AR up-regulation on non-peptidergic nociceptors after CCI, this could provide a potential direct link between increased α_1-AR expression on nociceptors and neuropathic pain.

α_1-AR expression was up-regulated on nerve fibers co-labelled with $CGRP^+$ or $IB4^+$, and was also up-regulated in nerve bundles on fibers co-labelled with NF200, a marker of myelinated nociceptive and non-nociceptive neurons. These nerve fibers may carry different types of pain information; $CGRP^+$ nociceptors respond to noxious heat and are partly responsible for hypersensitivity to heat after nerve injury [17, 30], whereas non-peptidergic nociceptors respond both to noxious heat and mechanical stimuli and contribute to heat hypersensitivity and mechanical allodynia after nerve injury [16, 41, 42, 45, 46]. Similarly, decreases in the firing threshold of myelinated nociceptors may increase the intensity of sharp, pricking pain after nerve injury [8]. Therefore, if α_1-AR expression increases the excitability of these nociceptors, α_1-AR up-regulation could potentially contribute to symptoms of neuropathic pain.

α_1-AR expression may also influence pain signalling indirectly by stimulating the release of secondary mediators that act on nociceptors. α_1-AR expression was increased throughout the epidermis in plantar skin affected by CCI. The normal epidermis contains nerve fibers, Langerhans cells, melanocytes, and keratinocytes. Melanocytes and Langerhans cells are relatively rare and are confined almost exclusively to the basal (germinative) layer, whereas keratinocytes are present in all the vital layers. Keratinocytes can influence nerve signalling by releasing various factors that are capable of activating and sensitizing nerve fibers after injury including nerve growth factor (NGF), pro-inflammatory cytokines, CGRP and ATP [15, 23, 24, 38, 40, 53]. Given the up-regulation of α_1-AR expression in the CCI-affected plantar epidermis, it could be hypothesised that activation of these receptors might cause release of factors from keratinocytes and/or other epidermal cells that are capable of stimulating nociceptive signalling, resulting in sensitization of these nociceptive nerve fibers and contributing to neuropathic pain. The release of NGF could be of particular importance as it might not only sensitize nociceptive afferents but could also promote the growth of re-innervating cutaneous sympathetic nerve fibers into the upper dermis after CCI; these fibers grow in close proximity to nociceptors and are a possible source of the norepinephrine needed to activate α_1-ARs [51]. Keratinocytes and melanocytes also have the capacity to synthesize catecholamines [13]. Thus, one could speculate that autocrine stimulation of α_1- or β_2-ARs[23] on these epidermal cells triggers a cascade of inflammatory mediators that augment neuropathic pain.

α_1-AR up-regulation was observed in plantar skin but not in hairy skin on the dorsal paw. This was an unexpected finding as both types of skin are innervated by branches of the sciatic nerve, and TUJ1 staining suggested a trend for neurodegeneration in both regions. Interestingly, in a previous study, neurons in hairy skin regenerated faster than neurons in plantar skin after sciatic nerve injury [39], suggesting that there may be some inherent difference between these regions that results in different responses to injury. Our finding that $NF200^+$ fibers were profoundly degenerated in plantar, but not hairy, skin supports this hypothesis. Extensive degeneration of myelinated fibers after CCI

has been observed previously [4, 32, 37]. However, this is the first study to compare the degeneration in plantar and hairy skin. Why there was a difference between the two types of skin in the response to nerve injury is not yet understood, but one major difference is that the plantar skin is completely innervated by branches of the sciatic nerve whereas the medial section of the dorsal hind paw is innervated by the saphenous nerve [47]. One could speculate that the presence of uninjured saphenous nerve fibers in the hairy skin could provide a neuroprotective effect, at least during the first week after injury (the only time point examined in this study). In addition, it would be interesting to determine whether a functional process (e.g., triggered by weight-bearing on the plantar surface of the paw[11]) influenced the response to CCI.

α_1-AR expression on large and small blood vessels was unaffected by CCI. This is interesting as blood vessels appear to have heightened sensitivity to α_1-AR agonists after injury [21, 49, 52]. This has resulted in the hypothesis that α_1-AR expression may be increased on blood vessels in cases of sympathetically-maintained pain and that α_1-AR activation causes pain by inducing vasoconstriction [48]. Our results suggest that increased vascular sensitivity to α_1-AR agonists after injury is not due to increased vascular expression of α_1-ARs, at least in the CCI model, but instead may be a direct consequence of denervation of blood vessels and consequent loss of neuronal norepinephrine transporters [44]. This could explain both the lack of α_1-AR up-regulation on blood vessels and increased sensitivity to α_1-AR agonists observed after CCI.

One limitation of this study was that α_1-AR expression was examined at only one time point, 7 days post-injury. This time point was chosen because previous studies have consistently reported the presence of mechanical and thermal hyperalgesia at this early post-injury time [5, 18, 35, 36]. However, it would be interesting in future studies to determine whether α_1-AR up-regulation is present at later time points. In addition, it would be useful to include sham operated animals in future studies to investigate possible nonspecific effects of surgery.

In conclusion, the up-regulation of α_1-ARs both in the epidermis and on nerve fibers in skin affected by CCI provides insights into the mechanism of involvement of the sympathetic nervous system in neuropathic pain. The increased expression of α_1-ARs after injury suggests that epidermal cells and nociceptive nerve fibers may become more sensitive to epinephrine and norepinephrine released as a result of sympathetic neural or adrenal gland excitation, or perhaps even synthesized locally within the epidermis. This could either directly activate α_1-ARs expressed on nociceptors or indirectly excite nociceptors by activation of epidermal cells and consequent release of factors that act on nociceptive nerve fibers.

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Figure Legends

Figure 1. Representative images of $NF200^+$ fibers in nerve bundles in the reticular dermis of plantar and hairy skin. Dashed lines identify the perimeter of nerve bundles. Scale bar = 50 μm

Figure 2. Representative images of α_1-AR expression in nerve bundles in the reticular dermis of plantar skin. α_1-AR expression was increased in nerve bundles in skin affected by CCI (lesioned) in comparison to skin on the contralateral limb to CCI (contra) and from naive animals. α_1-AR immunoreactivity co-localised with the pan-neuronal marker TUJ1. Co-localized pixels are shown in white in the co-localized panel of images. Scale bar = 50 μm

Figure 3. α_1-AR immunoreactivity (expressed as Z-scores ± S.E.) in dermal TUJ^+ nerve fibers and on epidermal cells on the injured and contralateral sides after chronic constriction injury (N = 6) and in naïve animals (N = 3). A-C: In plantar skin, α_1-AR immunoreactivity was greater on the injured than contralateral side in nerve fibers and on epidermal cells (* $p<0.05$), and was greater on the injured side than in uninjured naïve animals (#$p<0.05$). D-F: In the dorsal paw, α_1-AR immunoreactivity was similar in injured and naïve animals.

Figure 4. α_1-AR immunoreactivity (expressed as Z-scores ± S.E.) in TUJ^+ nerve fibers in plantar skin co-labelled with CGRP, IB4 or NF200 after chronic constriction injury (N = 6) and in naïve animals (N = 3). A: α_1-AR expression on fibers co-labelled with CGRP was greater on the injured than contralateral side (* $p<0.05$). B: α_1-AR expression on fibers co-labelled with IB4 was greater on the injured than contralateral side (* $p<0.05$), and was greater on the injured side than in uninjured naïve animals (#$p<0.05$). C: Trends were similar in nerve fibers co-labelled with NF200.

Figure 5. Representative images of α_1-AR expression in the epidermis. α_1-AR immunoreactivity was increased after CCI in plantar skin, but not hairy skin in the dorsal hind paw, in comparison to skin

contralateral to CCI and from naive animals. HF: indicates location of hair follicles in hairy skin. Scale bar = 100 μm

Figure 6. Representative image of α_1-AR expression in a dermal blood vessel stained with smooth muscle actin (SMA) (scale bar = 40 μm). α_1-AR immunoreactivity (expressed as Z-scores ± S.E.) on cells co-labelled with SMA was similar on the injured and contralateral sides after chronic constriction injury (N = 6) and in naïve animals (N = 3) both (A) in plantar and (B) hairy skin.

Table 1: Primary and secondary antibodies

Antibody	**Dilution**	**Product code and Source**
anti α_1-AR, rabbit polyclonal	1:200	A270, Sigma-Aldrich
anti BIII-tubulin (TUJ1), mouse monoclonal	1:800	MMS-435P, Covance
anti CGRP, goat polyclonal	1:400	1720-9007, AbD Serotec
IB4, FITC conjugate	1:250	L2895, Sigma-Aldrich
anti NF200, chicken polyclonal	1:4000	Jackson ImmunoResearch
anti-SMA, mouse monoclonal	1:4000	A2547, Sigma-Aldrich
anti-chicken Cy2	1:600	Jackson ImmunoResearch
anti-goat 488	1:600	Jackson ImmunoResearch
anti-rabbit 549	1:1200	Jackson ImmunoResearch
anti-mouse 647	1:1000	Jackson ImmunoResearch

Table 2: Proportion of TUJ1^{+} pixels within nerve bundles after CCI

	Mean ± S.E. (% of total area)		
	Naïve (N = 3)	CCI ipsilateral (N = 6)	CCI contralateral (N = 6)
Plantar skin	21±4	11±3	17±5
Dorsal skin	32±6	17±3	30±6

Table 3: Neural markers expressed as a proportion of dermal TUJ1^{+} pixels

	Mean ± S.E. (% of TUJ1^{+} pixels)		
	Naïve (N = 3)	CCI ipsilateral (N = 6)	CCI contralateral (N = 6)
Plantar skin			
CGRP	35±6	37±8	40±5
IB4	35±1	34±5	37±7
NF200	28±6	6±2 *	24±5
Dorsal skin			
CGRP	38±19	45±13	40±8
IB4	38±5	36±8	51±11
NF200	23±3	23±5	24±5

* $p<0.05$ compared with CCI contralateral and naïve animals