Published in final edited form as:

Pain. 2013 August ; 154(8): 1224–1236. doi:10.1016/j.pain.2013.03.033.

Epidermal adrenergic signaling contributes to inflammation and pain sensitization in a rat model of complex regional pain syndrome

Wenwu Li^{1,a,b}, Xiaoyou Shi^{1,a,b}, Liping Wang^a, Tianzhi Guo^a, Tzuping Wei^a, Kejun Cheng^c, Kenner C. Rice^c, Wade S. Kingery^a, and J. David Clark^{b,*}

^aPhysical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, CA

^bAnesthesiology Service, Veterans Affairs Palo Alto Health Care System Palo Alto, CA, and Department of Anesthesia, Stanford University School of Medicine, Stanford, CA

^cNational Institutes of Health/National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism

Keywords

Sympathetic signaling; interleukin-6; keratinocyte; complex regional pain syndrome

1. Introduction

The participation of the sympathetic nervous system (SNS) in complex regional pain syndrome (CRPS), once referred to as reflex sympathetic dystrophy, has long been appreciated. In 1916 Leriche proposed that sympathetic hyperactivity caused the development of posttraumatic CRPS, and he advocated surgical sympathectomy and sympathetic anesthetic nerve blocks for the treatment of this condition [28]. Sympathetic blocks are still a widely utilized treatment option for CRPS, but there is considerably controversy regarding the mechanisms by which SNS activity supports pain and the other components of CRPS [5]. Distal limb fracture is the most common cause of CRPS [7; 44; 56] and we have developed a rat tibia fracture/cast immobilization model that at 4 weeks post-fracture closely mimics the vascular, bony, nociceptive, and inflammatory changes observed in early CRPS [10; 59]. This model provides an opportunity to investigate the role of sympathetic signaling in persistent CRPS-like postfracture pain.

A large body of clinical evidence points to facilitated peripheral neurogenic inflammation, involving neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP), as contributing to some of the signs and symptoms of CRPS including pain sensitization, warmth and edema [2; 22; 27; 57]. Again, the fracture/cast CRPS model has provided results

*Correspondence should be addressed to J. David Clark, M.D., PhD., Anesthesia Service, Veterans Affairs Palo Alto Health Care System, 3801 Miranda Ave., Palo Alto, CA 94304, Tel: 650-493-5000 ext 67184, Fax: 650-852-3423, djclark@stanford.edu.

[†]These authors contributed equally to this paper

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of Interest Statement

The authors do not have financial or other relationships that might lead to conflict of interest.

consistent with the clinical findings; enhanced post-junctional facilitation of SP signaling was observed as was up-regulation of SP and CGRP in peripheral nerves, and expression of NK1 receptors [17; 58]. Furthermore, transgenic mice lacking functional SP or CGRP signaling had reduced post-fracture allodynia, unweighting, and vascular changes. These mice also failed to develop increased IL-1 β , TNF, and NGF in the hindpaw skin. Curiously, the transgenic mice did have increased IL-6 levels after fracture similar to the responses measured in control animals [12]. The up-regulation of IL-6 in CRPS may, therefore, occur via a distinct mechanism.

An increase in the abundance of IL-6 may be important in supporting CRPS. Levels of IL-6 are up-regulated in experimental skin blisters in CRPS limbs [14; 15] and we have observed IL-6, along with tumor necrosis factor alpha (TNF α), interleukin 1 β (IL-1), and nerve growth factor (NGF) are up-regulated in hindpaw skin at 4 weeks after tibia fracture in rats [41; 42; 59]. While effective, previous studies using anti-IL-1 β , TNF, and NGF agents provided only partial reversal of CRPS-like changes in the fracture/cast model [31; 41; 42]. On the other hand, it has been observed that intraplantar injection of IL-6 into normal hindpaw skin rapidly induces nociceptive sensitization [29]. Furthermore, it is known that keratinocytes express adrenoceptors and are capable of responding to norepinephrine, though the consequences of such stimulation have not been fully explored [39; 45; 50]. Beta-2-adrenergic receptors (β 2-AR's) appear to be the most abundantly expressed subtype *in vivo* [49]. We therefore hypothesized that SNS activity partially supports the inflammatory and nociceptive changes observed in the fracture/cast model of CRPS, and that norepinephrine released from sympathetic fibers acts through the stimulation of IL6 production to support these CRPS-like changes.

2. Materials and methods

These experiments were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, CA) and followed the animal subjects guidelines of the International Association for the Study of Pain. One hundred forty five adult (9-month-old) male Sprague-Dawley rats (Simonsen Laboratories, Gilroy, CA) were used in these experiments. The animals were housed individually in isolator cages with solid floors covered with 3 cm of soft bedding and were given food and water *ad libitum*. During the experimental period the animals were fed Lab Diet 5012 (PMI Nutrition Institute, Richmond, IN), which contains 1.0% calcium, 0.5% phosphorus, and 3.3 IU/g vitamin D3, and were kept under standard conditions with a 12-h light-dark cycle.

2.1. Surgery

Tibia fracture was performed under 2–4% isoflurane to maintain surgical anesthesia as we have previously described [10]. The right hind limb was wrapped in a stockinet (2.5cm wide) and the distal tibia was fractured using pliers with an adjustable stop (Visegrip, Petersen Manufacturing, Dewitt, NE) that had been modified with a three-point jaw. The hind limb was wrapped in casting tape ([delta]-Lite, Johnson & Johnson, New Brunswick, NJ) so the hip, knee, and ankle were flexed. The cast extended from the metatarsals of the hind paw up to a spica formed around the abdomen. The cast over the paw was applied only to the plantar surface; a window was left open over the dorsum of the paw and ankle to prevent constriction when post-fracture edema developed. To prevent the animals from chewing at their casts, the cast material was wrapped in galvanized wire mesh. The rats were given subcutaneous saline and buprenorphine immediately after procedure (0.03 mg/kg) and on the first day after fracture for postoperative hydration and analgesia. At 4 weeks the rats were anesthetized with isoflurane and the cast removed with a vibrating cast saw. All rats used in this study had union at the fracture site after 4 weeks of cast immobilization.

2.2. Drug treatment protocols

To test the hypothesis that sympathetic signaling can regulate cutaneous inflammation and nociceptive thresholds after tibia fracture, chemical sympathectomy was performed in fracture rats with either 6-hydroxydopamine (6-OHDA, Sigma) or guanethidine (Sigma). The 6-OHDA treatment was started at 7 days post-fracture using a progressive dosing schedule (50mg/kg/day 1, 50mg/kg/day 2, 100mg/kg/day 3, 100mg/kg/day 4, 100mg/kg/day 5, intraperitoneal injection, i.p.) that we had previously utilized to reduce cutaneous norepinephrine (NE) levels by 90% [19]. The guanethidine treatment was started at 14 days post-fracture and continued for 2 weeks (a total of 14 daily treatments, 50 mg/kg, subcutaneous injection, s.c., daily). This treatment protocol severely depletes NE levels in the peripheral sympathetic fibers [4; 37]. There were 2 sets of controls for these experiments, control rats that had no fracture or injections, and fracture control rats that were fractured/casted and received saline injections. Hindpaw nociceptive testing and assessment of warmth and edema were performed prior to fracture and at 4 weeks post-fracture (the day after cast removal). The next day the rats were euthanized and hindpaw skin was collected for EIA assays.

To test the hypothesis that β 2ARs mediate nociceptive and vascular changes in the CRPS model, rats underwent tibia fracture and were casted for 4 weeks, then the cast removed and the next day they were treated with a β 2AR antagonist, butoxamine (2mg/kg, intraperitoneal injection, i.p., Sigma). Hindpaw nociceptive testing and assessment of warmth and edema were performed prior to and 30 min after butoxamine injection. There were 2 sets of controls for these experiments, control rats that had no fracture or injections, and fracture control rats that were fractured/casted and received a saline injection. To further examine the pro-nociceptive effects of β 2AR activation in normal skin, the β 2AR agonist terbutaline was injected intradermally into the plantar hindpaw skin of normal control rats (5 μ g/30 μ l saline, intraplantar injection, i.pl., Sigma). Nociceptive testing was performed at baseline and at 0.5, 1, and 3 h after i.pl. terbutaline injection. In another group of rats the hindpaw skin was harvested 1 hour after terbutaline injection to determine if β 2AR activation up-regulated inflammatory cytokine and NGF production. Immunohistochemistry was used to identify the cellular origin of the up-regulated inflammatory mediators. The dosages of butoxamine and terbutaline used in the current study were based on previous reports of effective doses in another pain model [32].

To assess if IL-6 supported allodynia, unweighting, warmth, and edema in the CRPS model, rats underwent tibia fracture and were casted for 4 weeks, then the cast removed and the next day they were treated with TB-2-081 (2mg/kg, s.c., a generous gift from Dr Kenner Rice, NIDA) [20]. TB-2-081 is an orally active small molecule IL-6 receptor antagonist originally isolated from the skin of a toad. The TB-2-081 dosage used in the current study was based on a previous report of alleviation of chronic pancreatitis pain in rats with 1mg/kg, s.c. [55]. Hindpaw nociceptive testing and assessment of warmth and edema were performed prior to and 15 min after TB-2-081 injection. There were 2 sets of controls for these experiments, control rats that had no fracture or injections, and fracture control rats that were fractured/casted and received a saline injection.

2.3 Hindpaw nociception testing

To measure mechanical allodynia in the rats, an up-down von Frey testing paradigm was used as we have previously described [10; 11; 18]. Hind paw mechanical nociceptive thresholds were analyzed as the difference between the treatment side and the contralateral untreated side.

An incapacitance device (IITC Inc. Life Science, Woodland Hills, CA) was used to measure hind paw unweighting. The rats were manually held in a vertical position over the apparatus with the hind paws resting on separate metal scale plates, and the entire weight of the rat was supported on the hind paws. The duration of each measurement was 6s, and 10 consecutive measurements were taken at 60s intervals. Eight readings (excluding the highest and lowest) were averaged to calculate the bilateral hind paw weight bearing values [10; 11; 18] Right hindpaw weight bearing data were analyzed as a ratio between the right hindpaw weight and the sum of right and left hindpaws values $((2R/(R + L)) \times 100\%)$.

2.4. Hind paw volume testing

A laser sensor technique was used to determine the dorsal-ventral thickness of the hind paw, as we have previously described [10; 11; 18]. Hind paw volume data were analyzed as the difference between the treatment side and the contralateral untreated side.

2.5. Hindpaw temperature testing

For these experiments room temperature was maintained at 23°C and humidity ranged between 25% and 45%. The temperature of the hindpaw was measured using a fine wire thermocouple (Omega, Stamford, CT) applied to the paw skin, as previously described [10; 11; 18]. Hindpaw temperature data were analyzed as the difference between the treatment side and the contralateral untreated side.

2.6. Cell culture

The rat keratinocyte (REK) cell line was generously provided by Dr. Howard Baden (Massachusetts General hospital, Boston, MA) and grown as previously described [38]. In brief, cells were cultured in Dulbecco's modified Eagle's medium DMEM (Invitrogen) supplied with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin and 0.4µg/ml hydrocortisone (Sigma), 0.75mM aminoguanidine (Sigma) and passed weekly. REK were plated at 1×10^4 cells per 60 mm dish. Cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C and culture medium was changed every other day. On reaching approximately 80% confluence, the cells were treated with above culture medium supplemented with only 1% FBS for overnight before addition of substance P (SP, Sigma), norepinephrine (NE, Sigma), cirazoline (Sigma), dexmedetomidine (Sigma), dobutamine (Sigma) and terbutaline (Sigma).

2.7. Immunofluorescent cytochemistry

For antibody staining, cells cultured on coverslips were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 20 minutes at room temperature and then permeabilized with ice-cold ethanol for 5 min. Rat epidermal keratinocyte cell line were incubated with 5% FBS plus 1% bovine serum albumin at room temperature for 1 h to reduce background staining, then treated with primary antibody against β2AR (1:500, Abcam Inc., Cambridge, MA), rabbit α1 (1:1000, Thermo Scientific), rabbit β1, β3, goat α2A, α2B, or goat α2C (1:50, Santa Cruz Biotechnology) overnight at 4°C. Cells were then treated with Texas red (1:200, Santa Cruz Biotechnology) or cy3-conjugated secondary antibodies (1:400, Jackson ImmunoResearch Laboratories, Inc. West grove, PA) for 1h at room temperature. Control slides were stained with just the secondary antibody, which yields low intensity non-specific staining patterns. Immunostained cells were visualized with a Zeiss LSM 510 META laser scanning confocal microscope and the confocal software was used for acquisition of the data and merging of the digital images.

2.8. Quantitative real time PCR

Total RNA from cells growing in 60 mm culture dishes was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and the purity and concentration were determined spectrophotometrically. Next cDNA was synthesized from 1 µg RNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Real-time polymerase chain PCR reactions (PCRs) were conducted using the SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). Real-time PCR amplification of ARs (α 1A-AR, α 1B-AR, α 1D-AR, α 2A-AR, α 2BAR, α 2C-AR, β 1AR, β 2AR, and β 3-AR), IL-1 β , IL-6, TNF- α , NGF, and 18S was performed on an ABI 7900HT sequencing detection system. To validate the primer sets used, we performed dissociation curves to document single product formation, and agarose gel analysis was conducted to confirm the size. The data from real time PCR experiments were analyzed by the comparative CT method as described in the manual for the ABI prism 700 real time systems. All results were confirmed by repeating the experiment 3 times.

2.9. Enzyme immunoassay for TNF- α , IL-1 β , IL6 and NGF

Rat hindpaw dorsal skin was collected after behavioral testing or at time points as indicated and frozen immediately on dry ice. Skin tissue was cut into fine pieces in ice-cold phosphate buffered saline (PBS), pH 7.4, containing protease inhibitors (aprotinin (2 µg/ml) leupeptin (5 µg/ml), pepstatin (0.7 µg/ml), and PMSF (100 µg/ml); Sigma, St. Louis, MO, USA) followed by homogenization using a rotor/stator homogenizer. Homogenates were centrifuged for 5 min at 14 000 g, 4°C. Supernatants were transferred to fresh pre-cooled microcentrifuge tubes. Triton X-100 (Boehringer Mannheim, Germany) was added at a final concentration 0.01 %. The samples were centrifuged again for 5 min at 14 000g at 4°C. The supernatants were aliquoted and stored at -80°C. TNF- α , IL-1 β , and IL-6 protein levels were determined by using EIA kits (R&D Systems). The NGF concentrations were determined by using the NGF Emax® ImmunoAssay System kit (Promega, Madison, WI) according to the manufacturer's instructions. The OD of the reaction product was read on a microplate reader at 450 nm, and values were normalized per gram of tissue assayed. The concentrations of TNF- α , IL-1 β , IL-6, and NGF proteins were calculated from the standard curve prepared using reagents provided with each kit. Positive and negative controls were included in each assay as well. Total protein contents in all tissue extracts were measured by the Coomassie Blue Protein Assay Kit (Pierce, Rockford, IL). Each protein concentration was expressed as pg/mg total protein.

2.10. Western blot analysis

For western blot analysis of NF- κ B p65, nuclear extract was prepared as previously described [51]. Briefly, keratinocytes were washed with ice-cold PBS, scraped and briefly centrifuged. The cell pellet were then resuspended in a hypotonic lysis buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazinethansulfonic acids (HEPES), 1.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT), 0.5 µg/ml leupeptin, and 6.4% Nonidet P-40 and incubated for 15 minutes on ice. After another brief centrifugation, the nuclear pellet was collected and suspended in nuclear extraction buffer containing 20 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM 4-(2- aminoethyl) benzenesulfonylfluoride, 5 µg/ml pepstatin A and 5 µg/ml leupeptin. After incubation on ice for 30 minutes, the nuclear extract was collected; the concentration of protein was measured using a DC Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein were boiled with 3X sodium dodecyle sulfate (SDS) sample buffer, and then subjected to SDS electrophoresis. The results of this assay were confirmed by repeating the experiment 3 times.

For western blot analyses of p38, ERK, JNK, phosphorylated p38, phosphorylated ERK, and phosphorylated JNK, cultured keratinocytes were harvested and homogenized in Tris buffer

pH 6.8 with freshly added protease inhibitors, β -mercaptoethanol, and glycerol. The homogenate was centrifuged at 13,000 g for 15 minutes at 4°C. The concentration of protein was measured by using a DC Protein Assay Kit (Bio-Rad Laboratories). Equal amounts of protein were size fractionated by 10% SDS-PAGE gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The blot was blocked for overnight in 5% non-fat dry milk in Trisbuffered saline with 0.5% tween-20 (TBST), and incubated with primary antibody for detecting total p38 (1:1000, Cell Signaling Technology, Danvers, MA), total ERK1/2 (1:200, Santa Cruz Biotechnology), total JNK (1:400, Santa Cruz Biotechnology), phosphorylated p38 (1:1000, Cell Signaling Technology), phosphorylated ERK1/2 (1:200, Santa Cruz Biotechnology), phosphorylated JNK (1:400, Santa Cruz Biotechnology) or mouse NF- κ B p65 (1:200, Santa Cruz Biotechnology) on a rocking platform at 4°C for 24 h. After washing in TBST, the blots were incubated in horseradish peroxidase conjugated anti-rabbit or anti-mouse antibody (1:5000, Santa Cruz Biotechnology) for 1 h at room temperature. The membrane was then washed again and exposed to film following chemiluminescence reagent treatment with the ECL plus western blotting reagents (Amersham, Piscataway, NJ). Bands were quantified using densitometry of digitalized images. Each blot was then stripped and re-probed with anti- β actin antibody, thus allowing normalization of expression between samples. We assured both that the quantified bands were of the predicted molecular weights and that omission of primary antibodies eliminated the appearance of bands.

2.11. Immunohistochemistry and confocal microscopy

Animals were euthanized and immediately perfused with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), pH 7.4, via the ascending aorta; the hindpaw skin including sub-dermal layers was removed and post-fixed in 4% PFA for 2 hr, and then the tissues were treated with 30% sucrose in PBS at 4°C before embedding in OCT (Sakura Finetek). Following embedding, 10- μ m thick slices were made using a cryostat, mounted onto Superfrost microscope slides (Fisher Scientific), and stored at -80°C. To examine the effects of intraplantar terbutaline on IL-6 expression in epidermal keratinocytes, double immunolabeling was performed as previously described [29]. Briefly, frozen skin sections were permeabilized and blocked with PBS containing 10% donkey serum and 0.3% Triton X-100, followed by exposure to a rabbit anti-IL6 primary antibody (LifeSpan Biosciences, Inc., 1:400) overnight at 4 °C in PBS containing 2% serum. Sections were then rinsed in PBS and incubated with donkey anti-goat IgG (1:500) conjugated with fluorescein (FITC) secondary antibody (Jackson ImmunoResearch Laboratories). Upon detection of the IL-6, the sections were exposed to monoclonal anti-rat keratin (clone AE1/AE3, Thermo Fisher Scientific, diluted 1:50) overnight at 4°C in PBS containing 2% serum, and then the keratin was detected using donkey anti-mouse IgG (1:500) conjugated with cyanine dye 3 (Jackson ImmunoResearch Laboratories, diluted 1:500). Images were obtained using confocal microscopy (Zeiss LSM/510 Upright 2 photon; Carl Zeiss) and stored on digital media. Control experiments included incubation of slices in primary and secondary antibody-free solutions which led to low intensity non-specific staining patterns in preliminary experiments (data not shown). Pre-absorption controls were also completed. For these experiments, 250–500 ng of the primary antibody incubated with 2.5–6 μ g of the corresponding immunizing antigen in 500 μ L of PBS at 4°C overnight, before incubation with slices. These experiments also showed only low level staining.

2.12. Statistical analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) followed by post hoc Newman-Keuls multiple-comparison testing to compare effects of each drug (i.e., 6-OHDA, butoxamine, TB-2-081, and terbutaline) on nociceptive, vascular, or inflammatory mediator changes after tibia fracture with vehicle treatment and naïve control

group. Behavioral data collected over time after intraplantar injections of terbutaline were analyzed by a two-way ANOVA on data for each test time point, comparing with vehicle treatments. For simple comparisons of two means, unpaired Student t test was performed. For *in vitro* (keratinocyte culture) experiments, one-way ANOVA followed by post hoc Newman-Keuls test was performed to compare dose responses of each drug (i.e., norepinephrine, cirazoline, dexmedetomidine, dobutamine, and terbutaline). All data are presented as the mean $\pm$ standard error of the mean, and differences are considered significant at a P value less than 0.05 (Prism 5, GraphPad Software, San Diego, CA).

3. Results

3.1. Blockade of sympathetic signaling reduced inflammation, nociceptive sensitization and IL-6 production

To test the hypothesis that sympathetic signaling supports hindpaw pain and inflammation in the CRPS tibia fracture model, fracture rats with cast immobilization were treated with either 6-hydroxydopamine (6-OHDA) to abolish sympathetic nerve terminals starting 7 days after fracture and continuing for 5 days, guanethidine to deplete norepinephrine (NE) from sympathetic nerve terminals once daily starting 14 day after fracture for 2 weeks, or saline vehicle. We observed that blockade of sympathetic signaling by 6-OHDA reduced mechanical allodynia, hindlimb unweighting, warmth, and edema observed 4 weeks post-fracture (Fig. 1A–1D). As determined by EIA, 6-OHDA treatment also reduced skin IL-6 protein levels, but had no effects on IL-1 β , TNF α , and NGF (Fig. 1E–1H). Similar patterns were seen after guanethidine treatment. Guanethidine treatment reduced allodynia by 44% ($p < 0.01$), unweighting by 26% ($P < 0.05$), and decreased IL-6 by 47% ($p < 0.001$). These results suggest that sympathetic signaling contributes to inflammation, nociceptive sensitization and the post-fracture increased in IL-6.

3.2. Keratinocytes express adrenergic receptors (ARs) and the expression of those receptors was increased by norepinephrine

Because keratinocytes are the primary cellular source for the inflammatory mediators supporting cutaneous nociceptive sensitization in early CRPS [29], we were interested to know if sympathetic signals can support keratinocyte activation and inflammatory mediator production, thus maintaining nociceptive sensitization, allodynia and unweighting. To test this hypothesis, we first studied AR expression in cultured rat keratinocytes. The expression of ARs was determined by real-time PCR and later confirmed by immunofluorescence staining. Figure 2A demonstrates that mRNAs of all AR subtypes were detectable in cultured rat epidermal keratinocytes, but the AR β 2 subtype was expressed at much higher levels than any other subtypes. Immunofluorescence staining demonstrated the expression of ARs α 1 (Fig. 2B), α 2A (Fig. 2C), α 2B (Fig. 2D), α 2C (Fig. 2E), β 1 (Fig. 2F), β 2 (Fig. 2G) and β 3 (Fig. 2H) in the cultured cells.

We then examined the effects of norepinephrine (NE) on AR expression in cultured rat epidermal keratinocytes by real-time PCR. Norepinephrine increased the expression of all AR subtypes, except α 2A-AR and β 1-AR (Fig. 3). The stimulation effects of NE were maximal at 10^{-7} M for β 2AR, 10^{-6} M for α 2B-AR, and 10^{-5} M for α 1A-AR, α 1B-AR, α 1D-AR, α 2C-AR and β 3-AR measured at 3 h after NE addition (Fig. 3).

3.3. Activation of ARs by NE and various AR subtype agonists stimulated inflammatory mediator expression in keratinocytes

Next we looked at the effectiveness of NE and various AR subtype selective agonists in stimulating rat epidermal keratinocyte expression of IL-1, IL-6, TNF, and NGF by real-time PCR. As showed in figure 4, for all the agents we tested effects were generally dose-

dependent. The endogenous sympathetic neurotransmitter NE stimulated expression of all 4 inflammatory mediators, but the most robust effect was on IL-6 (Fig. 4A, Fig. 3-fold increase). Cirazoline (α 1-AR agonist) increased expression of all 4 inflammatory mediators, up to 2-fold (Fig. 4E–4H). Dexmedetomidine (α 2-AR agonist) increased expression of IL-1, up to 2-fold (Fig. 4J). Dobutamine (β 1-AR agonist) increased expression of IL-6, IL-1, and NGF by up to 50% (Fig. 4M, 4N, and 4P). Terbutaline (β 2AR agonist) increased expression of IL-6 by 18-fold, IL-1 by 2-fold, and NGF by 50% (Fig. 4Q, 4R, and 4T). Taken together, these results suggest that activation of ARs by NE and various AR subtype agonists stimulated inflammatory mediator expression in keratinocytes. However, enhanced IL-6 production via the stimulation of β 2ARs is by far the most robust response.

3.4. Norepinephrine (NE) stimulates phosphorylation of MAP kinases P38, ERK, and JNK

We sought to investigate the effectiveness of NE in stimulating phosphorylation of MAP kinases p38, ERK, and JNK, as well as nuclear translocation of NF κ B in keratinocytes by Western blots. Figure 5 demonstrated that the application of NE (10^{-5} M) caused peak phosphorylation of all 3 MAPKs at 5 min. However, NE did not affect nuclear translocation of NF- κ B in rat keratinocytes.

3.5. Blockade of β 2AR by butoxamine in fracture rats reduced allodynia, unweighting and warmth, but had no effect on edema

Given that the β 2AR subtype is predominant and functional in keratinocytes and that sympathetic signaling supports inflammation and pain sensitization, we hypothesized β 2AR receptors mediate nociceptive changes observed in the CRPS model. To test this hypothesis, fracture rats and non-injured animals were treated with systemic administered β 2AR antagonist, butoxamine (2 mg/kg i.p. 30 min prior to testing). As showed in figure 6, butoxamine treatment reduced hind paw allodynia and hindlimb unweighting that developed after fracture. However, it didn't change hindpaw warmth as well as edema. In non-injured animals, administration of butoxamine at the same dose did not change hindpaw nociceptive responses, edema and warmth (data not shown).

3.6. Blockade of the IL-6 receptor by TB-2-081 in fracture rats reduced allodynia, unweighting and warmth, but no effect on edema

IL-6 is an inflammatory mediator well known to support nociception including mechanical allodynia in skin [1; 29; 53]. The blockade of sympathetic signaling (Fig. 1) reduced inflammation, nociceptive responses and IL-6 production. This led us to hypothesize that sympathetic signaling may regulate nociceptive responses via IL-6 in CRPS, i.e., NE may promote IL-6 production and secretion from keratinocytes, which in turn acts directly or indirectly on sensory nerve terminals in the skin support allodynia and unweighting seen in the CRPS model.

To examine the hypothesis of IL-6 supporting nociceptive response in the CRPS model, tibia fracture rats and non-injured animals were treated with an IL-6 receptor antagonist, TB-2-081 (2mg/kg s.c. 15 min prior to testing or vehicle). We observed that treatment with TB-2-081 reduced hind paw allodynia and unweighting that developed after fracture. However, this agent did not significantly alter hindpaw warmth and edema (Fig. 7). In non-injured animals, administration of TB-2-081 at the same dose did not change hindpaw nociceptive responses, edema and warmth (data not shown).

3.7. Activation of β 2AR by terbutaline in control rats induced local mechanical allodynia and IL-6 production in keratinocytes

Because the systemic administration of butoxamine does not allow us to conclude that local hindpaw ARs are the population of ARs supporting peripheral inflammatory changes, we determined if terbutaline (a selective β 2AR agonist) applied locally could induce mechanical allodynia and IL-6 production in skin in normal control rats. Our behavioral studies indicated that intraplantar application of terbutaline (5 μ g/30 μ l) caused hindpaw mechanical allodynia and hindlimb unweighting at 1 hr after injection (Fig. 8A and 8B). When terbutaline was given systemically at the same dose used in the plantar hindpaw injection experiment (5 μ g/30 μ l saline, i.p.) there was no effect on nociceptive thresholds (data not shown). On the other hand, using EIA we detected that the injection of terbutaline induced a significant increase in IL-6 cytokine level (Fig. 8c) but did not change IL-1 β , TNF- α or NGF levels (Fig. 8D, 8E and 8F respectively) in skin. Figure 8G shows representative confocal images of IL-6 (green) and keratin (a keratinocyte marker, red) in hindpaw skin sections from control rats, and from the ipsilateral hindpaw of control rats treated with terbutaline. Only low levels of IL-6 were detected in control skin (Fig. 8G, upper row of panels), but skin sections from terbutaline injected rats demonstrated large amount of IL-6 protein in cells in the epidermis co-labeled for keratin (Fig. 8G, middle and bottom rows of panels).

4. Discussion

In this study we used two well-characterized strategies to reduce SNS activity in fracture rats, including administration of 6-OHDA, a catechol synthesis inhibitor, and guanethidine, an agent which depletes NE from sympathetic nerve terminals. Using these agents nociceptive changes were reduced and the vascular changes were partially normalized (Fig. 1). Because TNF- α and IL-6 levels are elevated in the skin blister fluid of CRPS affected limbs and TNF- α , IL-1 β , IL-6 and NGF levels are elevated at 4 weeks post-fracture in the tibia fracture/cast model of CRPS, we went on to examine the effects of sympathectomy on inflammatory mediator levels in the hindpaw skin after fracture. Curiously, we found that 6-OHDA or guanethidine treatment inhibited the expected increase in IL-6 levels in the fracture hindpaw skin, while levels of the other mediators were elevated at levels similar to those observed in untreated control animals after fracture (Fig. 1).

The effects of sympathectomy on post-fracture pain behavior and mediator expression correlates with our recent study examining the effects of fracture in transgenic mice lacking either SP or the CGRP receptor RAMP1 [12]. At 3 weeks post-fracture these mice had attenuated nociceptive sensitization compared to wildtype fracture mice, and while there were no increases in IL-1 β , TNF- α , or NGF levels in hindpaw skin, IL-6 levels were increased, suggesting that this cytokine is regulated by a distinct mechanism. We postulated that the residual sensitization observed in the transgenic fracture mice was attributable to the increased IL-6 levels. In fact, the IL-6 receptor antagonist TB-2-081 partially reversed allodynia and unweighting. Previously we demonstrated that fracture rats treated with the IL-1 receptor antagonist anakinra, the TNF inhibitor etanercept, or the NGF antibody tanezumab had attenuated allodynia and hindlimb unweighting at 4 weeks post-fracture [31; 41; 42]. Because these cytokine and NGF inhibitors are large molecules that don't cross the blood-brain barrier, we suspect that the pro-nociceptive effects of these mediators occur at the nociceptor level in the skin. Furthermore, we identified keratinocytes as the primary cellular source of IL-1 β , IL-6, TNF- α , and NGF in the fracture hindpaw [29]. Collectively, these results suggest that exaggerated neuropeptide signaling induces the up-regulation of IL-1 β , TNF- α , and NGF in cutaneous keratinocytes of the fracture limb, thus contributing to nociceptive sensitization. Similarly, the sympathectomy experiments indicate that SNS

signaling contributes to postfracture nociceptive sensitization and to the up-regulation of IL-6 in keratinocytes.

A rat keratinocyte cell line was then employed to identify the adrenergic receptor populations responsible for the sympathetic regulation of inflammatory mediator expression and to study potential mechanisms for those effects [48]. While the expression of many adrenergic receptor subtypes was identified in these cells, β 2AR expression was remarkably high (Fig. 2). Expression of this receptor, like several other types of adrenergic receptors, was enhanced after exposing keratinocytes to NE (Fig. 3). These results were complemented by expression studies looking at IL-1 β , IL-6, TNF- β , and NGF, all mediators found in elevated levels in the skin of fracture/cast animals [29]. Stimulation of the keratinocytes with NE increased the levels of several cytokines and NGF, though IL-6 was by far the most robustly stimulated (Fig. 4). Importantly, the highly selective β 2AR agonist terbutaline caused an 18-fold increase in IL-6 expression, more than an order of magnitude greater increase than for any of the other mediators studied. It should be noted that selective stimulation of other classes of adrenergic receptors did in fact lead to measurable increases in cytokine levels, though the increases were typically less dramatic than the terbutaline mediated effects on IL-6. Specifically, the 4 mediators followed in these experiments were all increased after exposure of the cultures to the selective α 1AR agonist cirazoline.

In addition, stimulation of keratinocytes with terbutaline activated all three of the principal families of the MAPK signaling system, but not the NF- κ B transcriptional signaling pathway (Fig. 5). The MAPK signaling system has been implicated in promoting cytokine production (IL-1 β , IL-6, TNF- α ,) in many tissues, including skin [25; 35; 47]. Particularly strong evidence relates p38 MAPK activation to IL-6 production in keratinocytes, which is consistent with our observations [26]. In our experiments phosphorylation of MAPK-related enzymes was observed within 5 minutes of exposure of keratinocyte cultures to NE. Strong evidence also links activation of the NF- κ B system to cytokine production in various types of cells [3; 24]. This, however, was not observed in our system. We did not attempt to determine whether these signaling systems were activated under conditions *in vivo*.

These *in vitro* observations were then translated to the animal model. As predicted from the cell culture results, systemic administration of the selective β 2AR antagonist butoxamine reduced nociceptive sensitization in the fracture/cast animals (Fig. 6). These results closely parallel those obtained when chemical sympathectomy was performed. Furthermore, the systemic administration of the selective IL-6 receptor antagonist TB-2-081 also reduced hindpaw nociceptive sensitization in the fracture rats (Fig. 7). Complementing those observations, the intradermal injection of terbutaline in the hindpaw caused mechanical allodynia, hindpaw unweighting, and up-regulated the production of IL-6 (but not other mediators) in the keratinocyte cell layer of hindpaw skin (Fig. 8). Previous studies involving the local administration of SP showed a more generalized increase in cytokine and NGF levels in paw skin [43] suggesting that specific transmitters generate specific patterns of mediator production. Thus the keratinocyte β 2AR/IL6 regulatory axis could be reproduced in whole animals, adding credence to the notion that this axis is functional in causing some of the changes characterizing CRPS.

Our observation that β 2ARs are the predominant adrenergic receptor population expressed in cultured keratinocytes is consistent with existing literature. Though many subtypes of adrenergic receptors exist, keratinocytes predominantly express β 2AR's *in vivo* [49], especially those in the basal layer of the epidermis where we observed the majority of the IL-6 expression in our experiments [46]. We observed apparent up-regulation of β 2AR expression in response to NE in cell culture experiments, though the dose-response curve was U-shaped. This may be due to receptor desensitization, internalization, uncoupling or

depletion of second messengers [9]. Actual changes in functional expression were not assessed and require formal binding studies. These receptors have well established roles in controlling proliferation and differentiation of keratinocytes through control of cAMP and calcium ion levels [13; 33]. The mechanism for β 2AR stimulation on IL-6 production in epithelial cells is less well understood, though β 2AR stimulation causes the enhanced production of IL-6 in airway epithelial cells [8; 52]. The evoked release of norepinephrine in skin is similar between CRPS and control subjects suggesting that target tissue changes in adrenergic signaling may be important [54]. It is also noteworthy that catecholamines released from sympathetic nerve terminals may not be the only source of these transmitters in skin. Pullar et al. have reported that keratinocytes synthesize and release epinephrine, and that β 2AR's expressed on these keratinocytes may regulate cell proliferation in response to this epinephrine release [40]. We did not in these studies examine the question of increased adrenergic sensitivity of afferent neurons which may support pain in conditions such as Varicella zoster infection [23].

These data along with others previously reported can be assembled into an integrative model of neurocutaneous signaling in CRPS. Figure 9 updates our previously proposed model to accommodate sympathetic signaling [30]. Any such model needs to take into account the demonstrated functions of primary afferent fibers, as well as sympathetic input. The main demonstrated sources of cutaneous cytokines and NGF in this model are the keratinocytes [29]. As diagramed in Figure 9, the neuropeptides SP and CGRP stimulate keratinocyte production of several different inflammatory mediators, including IL-1 β , TNF- α and NGF. In parallel, the SNS likely has a predominant, though perhaps not exclusive role in stimulating IL-6 production via β 2ARs activation on epidermal keratinocytes. All these inflammatory mediators can cause rapid cutaneous nociceptive sensitization after intraplantar injection [29] and can immediately evoke spontaneous firing and sensitization in primary sensory neurons at the nociceptors level [6; 16; 36]. We suggest that partially overlapping neurocutaneous mechanisms support the production of nociceptive mediators in CRPS, and that the relative balance of these systems may explain in part the heterogeneous clinical presentation and differences in treatment responses in the CRPS patient population.

In conclusion, the results from the keratinocyte *in vitro* studies and the rat fracture CRPS model support our hypothesis that the release of NE from the sympathetic terminals in the skin can activate β 2ARs on epidermal keratinocytes, resulting in the production and secretion of the pro-inflammatory cytokine IL-6, subsequently causing sensitization of cutaneous sensory neuron terminals with allodynia and unweighting in the fracture limb. We postulate that adrenergic keratinocyte-sensory neuron neuroinflammatory signaling also plays a role in the modulation of nociceptive thresholds in CRPS patients. Previous reports that CRPS patients frequently express β 2AR antibodies that can act as β 2AR agonists [21] and that IL-6 levels are up-regulated in experimental skin blister fluid in the CRPS affected limb [14; 34] further support to this hypothesis. Collectively, these data support mechanism-based pharmacologic trials examining the role of β 2AR signaling and IL-6 immune responses in early CRPS.

Acknowledgments

This study was supported by National Institutes of Health grants NS072168 and NS072143, and Department of Veterans Affairs, Rehabilitation Research and Development Merit grants F7137R. A portion of this work was supported by the Intramural Research Programs of National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism.

References

1. Asiedu MN, Tillu DV, Melemedjian OK, Shy A, Sanoja R, Bodell B, Ghosh S, Porreca F, Price TJ. Spinal protein kinase M zeta underlies the maintenance mechanism of persistent nociceptive sensitization. *J Neurosci*. 2011; 31(18):6646–6653. [PubMed: 21543593]
2. Birklein F, Schmelz M. Neuropeptides, neurogenic inflammation and complex regional pain syndrome (CRPS). *Neuroscience letters*. 2008; 437(3):199–202. [PubMed: 18423863]
3. Blackwell TS, Christman JW. The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol*. 1997; 17(1):3–9. [PubMed: 9224203]
4. Borghini N, Dalmaz Y, Peyrin L. Effect of guanethidine on dopamine in small intensely fluorescent cells of the superior cervical ganglion of the rat. *J Auton Nerv Syst*. 1991; 32(1):13–19. [PubMed: 1673696]
5. Bruhl S. An update on the pathophysiology of complex regional pain syndrome. *Anesthesiology*. 2010; 113(3):713–725. [PubMed: 20693883]
6. De Jongh RF, Vissers KC, Meert TF, Booij LH, De Deyne CS, Heylen RJ. The role of interleukin-6 in nociception and pain. *Anesth Analg*. 2003; 96(4):1096–1103. table of contents. [PubMed: 12651667]
7. de Mos M, de Bruijn AG, Huygen FJ, Dieleman JP, Stricker BH, Sturkenboom MC. The incidence of complex regional pain syndrome: a population-based study. *Pain*. 2007; 129(1–2):12–20. [PubMed: 17084977]
8. Edwards MR, Haas J, Panettieri RA Jr, Johnson M, Johnston SL. Corticosteroids and beta2 agonists differentially regulate rhinovirus-induced interleukin-6 via distinct Cis-acting elements. *The Journal of biological chemistry*. 2007; 282(21):15366–15375. [PubMed: 17395587]
9. Emerich DF, Snodgrass P, Dean RL, Lafreniere D, Agostino M, Wiens T, Xiong H, Hasler B, Marsh J, Pink M, Kim BS, Bartus RT. Bradykinin modulation of tumor vasculature: I. Activation of B2 receptors increases delivery of chemotherapeutic agents into solid peripheral tumors, enhancing their efficacy. *J Pharmacol Exp Ther*. 2001; 296(2):623–631. [PubMed: 11160651]
10. Guo TZ, Offley SC, Boyd EA, Jacobs CR, Kingery WS. Substance P signaling contributes to the vascular and nociceptive abnormalities observed in a tibial fracture rat model of complex regional pain syndrome type I. *Pain*. 2004; 108(1–2):95–107. [PubMed: 15109512]
11. Guo TZ, Wei T, Kingery WS. Glucocorticoid inhibition of vascular abnormalities in a tibia fracture rat model of complex regional pain syndrome type I. *Pain*. 2006; 121(1–2):158–167. [PubMed: 16472917]
12. Guo TZ, Wei T, Shi X, Li WW, Hou S, Wang L, Tsujikawa K, Rice KC, Cheng K, Clark DJ, Kingery WS. Neuropeptide deficient mice have attenuated nociceptive, vascular, and inflammatory changes in a tibia fracture model of complex regional pain syndrome. *Mol Pain*. 2012; 8:85. [PubMed: 23191958]
13. Hennings H, Michael D, Cheng C, Steinert P, Holbrook K, Yuspa SH. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell*. 1980; 19(1):245–254. [PubMed: 6153576]
14. Huygen FJ, De Bruijn AG, De Bruin MT, Groeneweg JG, Klein J, Zijlstra FJ. Evidence for local inflammation in complex regional pain syndrome type I. *Mediators Inflamm*. 2002; 11(1):47–51. [PubMed: 11930962]
15. Huygen FJ, Ramdhani N, van Toorenenbergen A, Klein J, Zijlstra FJ. Mast cells are involved in inflammatory reactions during Complex Regional Pain Syndrome type I. *Immunol Lett*. 2004; 91(2–3):147–154. [PubMed: 15019283]
16. Jin X, Gereau RWt. Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-alpha. *J Neurosci*. 2006; 26(1):246–255. [PubMed: 16399694]
17. Kingery WS. Role of neuropeptide, cytokine, and growth factor signaling in complex regional pain syndrome. *Pain medicine*. 2010; 11(8):1239–1250. [PubMed: 20704672]
18. Kingery WS, Davies MF, Clark JD. A substance P receptor (NK1) antagonist can reverse vascular and nociceptive abnormalities in a rat model of complex regional pain syndrome type II. *Pain*. 2003; 104(1–2):75–84. [PubMed: 12855316]

19. Kingery WS, Guo TZ, Davies MF, Limbird L, Maze M. The alpha2 adrenoceptor and the sympathetic postganglionic neuron contribute to the development of neuropathic heat hyperalgesia in mice. *Pain*. 2000; 85:345–358. [PubMed: 10781908]
20. Kino T, Boos TL, Sulima A, Siegel EM, Gold PW, Rice KC, Chrousos GP. 3-O-Formyl-20R,21-epoxyresibufogenin suppresses IL-6-type cytokine actions by targeting the glycoprotein 130 subunit: potential clinical implications. *J Allergy Clin Immunol*. 2007; 120(2):437–444. [PubMed: 17451794]
21. Kohr D, Singh P, Tschernatsch M, Kaps M, Pouokam E, Diener M, Kummer W, Birklein F, Vincent A, Goebel A, Wallukat G, Blaes F. Autoimmunity against the beta(2) adrenergic receptor and muscarinic-2 receptor in complex regional pain syndrome. *Pain*. 2011; 152(12):2690–2700. [PubMed: 21816540]
22. Kramer HH, Eberle T, Uceyler N, Wagner I, Klonschinsky T, Muller LP, Sommer C, Birklein F. TNFalpha in CRPS and 'normal' trauma--significant differences between tissue and serum. *Pain*. 2011; 152(2):285–290. [PubMed: 20947251]
23. Kress M, Fickenscher H. Infection by human varicella-zoster virus confers norepinephrine sensitivity to sensory neurons from rat dorsal root ganglia. *FASEB J*. 2001; 15(6):1037–1043. [PubMed: 11292665]
24. Lappas M, Permezel M, Georgiou HM, Rice GE. Nuclear factor kappa B regulation of proinflammatory cytokines in human gestational tissues in vitro. *Biol Reprod*. 2002; 67(2):668–673. [PubMed: 12135912]
25. Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature*. 1994; 372(6508):739–746. [PubMed: 7997261]
26. Lee JW, Ryu HC, Ng YC, Kim C, Wei JD, Sabaratnam V, Kim JH. 12(S)-Hydroxyheptadeca-5Z, 8E, 10Etrienoic acid suppresses UV-induced IL-6 synthesis in keratinocytes, exerting an antiinflammatory activity. *Exp Mol Med*. 2012
27. Leis S, Weber M, Isselmann A, Schmelz M, Birklein F. Substance-P-induced protein extravasation is bilaterally increased in complex regional pain syndrome. *Experimental neurology*. 2003; 183(1):197–204. [PubMed: 12957502]
28. Leriche R. De la causalgia envisagee comme une nevrite du sympathique et de son traitement par la denudation et l'excision des plexus nerveux peri-arteriels. *Press Med*. 1916; 24:178–180.
29. Li WW, Guo TZ, Li XQ, Kingery WS, Clark JD. Fracture induces keratinocyte activation, proliferation, and expression of pro-nociceptive inflammatory mediators. *Pain*. 2010; 151(3):843–852. [PubMed: 20934254]
30. Li WW, Guo TZ, Liang D, Shi X, Wei T, Kingery WS, Clark JD. The NALP1 inflammasome controls cytokine production and nociception in a rat fracture model of complex regional pain syndrome. *Pain*. 2009; 147(1–3):277–286. [PubMed: 19853379]
31. Li WW, Sabovich I, Guo TZ, Zhao R, Kingery WS, Clark JD. The role of enhanced cutaneous IL-1beta signaling in a rat tibia fracture model of complex regional pain syndrome. *Pain*. 2009; 144(3):303–313. [PubMed: 19473768]
32. Liang DY, Liao G, Wang J, Usuka J, Guo Y, Peltz G, Clark JD. A genetic analysis of opioid-induced hyperalgesia in mice. *Anesthesiology*. 2006; 104(5):1054–1062. [PubMed: 16645459]
33. Mammone T, Marenus K, Maes D, Lockshin RA. The induction of terminal differentiation markers by the cAMP pathway in human HaCaT keratinocytes. *Skin pharmacology and applied skin physiology*. 1998; 11(3):152–160. [PubMed: 9745142]
34. Munnikes RJ, Muis C, Boersma M, Heijmans-Antonissen C, Zijlstra FJ, Huygen FJ. Intermediate stage complex regional pain syndrome type 1 is unrelated to proinflammatory cytokines. *Mediators Inflamm*. 2005; 2005(6):366–372. [PubMed: 16489257]
35. Muthusamy V, Piva TJ. The UV response of the skin: a review of the MAPK, NFkappaB and TNFalpha signal transduction pathways. *Arch Dermatol Res*. 2010; 302(1):5–17. [PubMed: 19756672]
36. Pezet S, McMahon SB. Neurotrophins: mediators and modulators of pain. *Annu Rev Neurosci*. 2006; 29:507–538. [PubMed: 16776595]

37. Picklo MJ. Methods of sympathetic degeneration and alteration. *J Auton Nerv Syst.* 1997; 62(3): 111–125. [PubMed: 9051618]
38. Pirrone A, Hager B, Fleckman P. Primary mouse keratinocyte culture. *Methods Mol Biol.* 2005; 289:3–14. [PubMed: 15502164]
39. Pullar CE, Manabat-Hidalgo CG, Bolaji RS, Isseroff RR. beta-Adrenergic receptor modulation of wound repair. *Pharmacological research : the official journal of the Italian Pharmacological Society.* 2008; 58(2):158–164. [PubMed: 18790719]
40. Pullar CE, Rizzo A, Isseroff RR. beta-Adrenergic receptor antagonists accelerate skin wound healing: evidence for a catecholamine synthesis network in the epidermis. *The Journal of biological chemistry.* 2006; 281(30):21225–21235. [PubMed: 16714291]
41. Sabsovich I, Guo TZ, Wei T, Zhao R, Li X, Clark DJ, Geis C, Sommer C, Kingery WS. TNF signaling contributes to the development of nociceptive sensitization in a tibia fracture model of complex regional pain syndrome type I. *Pain.* 2008; 137(3):507–519. [PubMed: 18035493]
42. Sabsovich I, Wei T, Guo TZ, Zhao R, Shi X, Li X, Yeomans DC, Klyukinov M, Kingery WS, Clark JD. Effect of anti-NGF antibodies in a rat tibia fracture model of complex regional pain syndrome type I. *Pain.* 2008; 138(1):47–60. [PubMed: 18083307]
43. Sahbaie P, Shi X, Guo TZ, Qiao Y, Yeomans DC, Kingery WS, Clark JD. Role of substance P signaling in enhanced nociceptive sensitization and local cytokine production after incision. *Pain.* 2009; 145(3):341–349. [PubMed: 19660865]
44. Sandroni P, Benrud-Larson LM, McClelland RL, Low PA. Complex regional pain syndrome type I: incidence and prevalence in Olmsted county, a population-based study. *Pain.* 2003; 103(1–2): 199–207. [PubMed: 12749974]
45. Schallreuter KU. Epidermal adrenergic signal transduction as part of the neuronal network in the human epidermis. *The journal of investigative dermatology Symposium proceedings / the Society for Investigative Dermatology, Inc [and] European Society for Dermatological Research.* 1997; 2(1):37–40.
46. Schallreuter KU, Wood JM, Pittelkow MR, Swanson NN, Steinkraus V. Increased in vitro expression of beta 2-adrenoceptors in differentiating lesional keratinocytes of vitiligo patients. *Archives of dermatological research.* 1993; 285(4):216–220. [PubMed: 8393648]
47. Schett G, Zwerina J, Firestein G. The p38 mitogen-activated protein kinase (MAPK) pathway in rheumatoid arthritis. *Ann Rheum Dis.* 2008; 67(7):909–916. [PubMed: 17827184]
48. Shi X, Wang L, Li X, Sahbaie P, Kingery WS, Clark JD. Neuropeptides contribute to peripheral 'nociceptive sensitization by regulating interleukin-1beta production in keratinocytes. *Anesth Analg.* 2011; 113(1):175–183. [PubMed: 21596883]
49. Sivamani RK, Lam ST, Isseroff RR. Beta adrenergic receptors in keratinocytes. *Dermatologic clinics.* 2007; 25(4):643–653. x. [PubMed: 17903623]
50. Sivamani RK, Pullar CE, Manabat-Hidalgo CG, Rocke DM, Carlsen RC, Greenhalgh DG, Isseroff RR. Stress-mediated increases in systemic and local epinephrine impair skin wound healing: potential new indication for beta blockers. *PLoS medicine.* 2009; 6(1):e12. [PubMed: 19143471]
51. Sohn EJ, Kang DG, Choi DH, Lee AS, Mun YJ, Woo WH, Kim JS, Lee HS. Effect of methanol extract of *Sorbus cortex* in a rat model of L-NAME-induced atherosclerosis. *Biol Pharm Bull.* 2005; 28(7):1239–1243. [PubMed: 15997106]
52. Strandberg K, Palmberg L, Larsson K. Effect of formoterol and salmeterol on IL-6 and IL-8 release in airway epithelial cells. *Respiratory medicine.* 2007; 101(6):1132–1139. [PubMed: 17229563]
53. Summer GJ, Romero-Sandoval EA, Bogen O, Dina OA, Khasar SG, Levine JD. Proinflammatory cytokines mediating burn-injury pain. *Pain.* 2008; 135(1–2):98–107. [PubMed: 17590515]
54. Takahashi H, Kinouchi M, Tamura T, Iizuka H. Decreased beta 2-adrenergic receptor-mRNA and loricrin-mRNA, and increased involucrin-mRNA transcripts in psoriatic epidermis: analysis by reverse transcription-polymerase chain reaction. *The British journal of dermatology.* 1996; 134(6): 1065–1069. [PubMed: 8763426]
55. Vardanyan M, Melemedjian OK, Price TJ, Ossipov MH, Lai J, Roberts E, Boos TL, Deschamps JR, Jacobson AE, Rice KC, Porreca F. Reversal of pancreatitis-induced pain by an orally

- available, small molecule interleukin-6 receptor antagonist. *Pain*. 2010; 151(2):257–265. [PubMed: 20599324]
56. Veldman PHJM, Reynen HM, Arntz IE, Goris RJA. Signs and symptoms of reflex sympathetic dystrophy: prospective study of 829 patients. *Lancet*. 1993; 342:1012–1016. [PubMed: 8105263]
57. Weber M, Birklein F, Neundorfer B, Schmelz M. Facilitated neurogenic inflammation in complex regional pain syndrome. *Pain*. 2001; 91(3):251–257. [PubMed: 11275381]
58. Wei T, Li WW, Guo TZ, Zhao R, Wang L, Clark DJ, Oaklander AL, Schmelz M, Kingery WS. Postjunctional facilitation of Substance P signaling in a tibia fracture rat model of complex regional pain syndrome type I. *Pain*. 2009; 144(3):278–286. [PubMed: 19464118]
59. Wei T, Sabsovich I, Guo TZ, Shi X, Zhao R, Li W, Geis C, Sommer C, Kingery WS, Clark DJ. Pentoxifylline attenuates nociceptive sensitization and cytokine expression in a tibia fracture rat model of complex regional pain syndrome. *Eur J Pain*. 2009; 13(3):253–262. [PubMed: 18554967]

Fig. 1.

The effects of pharmacological blockade of sympathetic signaling on hindlimb nociception, vascular changes and pro-inflammatory cytokine production at 4 weeks after fracture. Top panels show intraperitoneal injection of 6-hydroxydopamine (6-OHDA) reduced post-fracture von Frey mechanical allodynia (A), unweighting (B), warmth (C), and edema (D). Measurements presented in panels A, C, and D represent the difference between the fracture side and the contralateral paw. Thus a positive value represents an increase in temperature or thickness on the fracture side, and a negative value represents a decrease in mechanical nociceptive threshold on the affected side. The data presented in panel B represents weight bearing on the fracture hindlimb with 100% representing an even distribution of weight between the fracture and contralateral hind paws. Bottom panels: Skin cytokine levels determined by EIA in hindpaw skin ipsilateral to tibia fracture. Treatment with 6-OHDA significantly decreased hindpaw skin IL-6 levels (E), but had no effect on IL-1 β (F), TNF- α (G), and NGF (H). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for FX-Vehicle (n=16) or FX-6OHDA (n=14) treatment vs. control group (n=16), # $P < 0.05$ and ## $p < 0.01$ for FX-6OHDA treatment (n=14) vs. FX-Vehicle (n=16). FX: fracture.

Fig. 2.

Adrenergic receptor (AR) expression in cultured rat epidermal keratinocytes. For all ARs expression was detectable at the mRNA level though the $\beta 2$ AR subtype was expressed at much higher levels than the others (A). Expression of each AR subtype was detectable at the protein level as well. Panels B – H are representative fluorescence photomicrographs of $\alpha 1$ -AR (B), $\alpha 2A$ (C), $\alpha 2B$ (D), $\alpha 2C$ (E), $\beta 1$ (F), $\beta 2$ (G) and $\beta 3$ (H).

Fig. 3. Effects of norepinephrine (NE) on adrenergic receptor (AR) expression in cultured rat epidermal keratinocytes measured using real-time PCR. Cells were harvested for analysis 3 h after NE addition. Expression of all AR subtypes were increased by NE (A, B, C, E, F, H and I), except α 2A -AR (D) and β 1-AR (G).

Fig. 4.

Effects of norepinephrine (NE) and various adrenergic receptor (AR) subtype selective agonists in stimulating keratinocyte expression of IL-6, IL-1 β , TNF- α and NGF in cultured rat epidermal keratinocytes by real-time PCR. The peak effects of NE were 10⁻⁵ M at 3 h after the addition of the agonist. NE stimulated expression of all 4 inflammatory mediators (A-D), but the most robust effect was on IL-6 (3-fold increase, A). Cirazoline (α 1-AR agonist) increased expression of all 4 inflammatory mediators, up to 2-fold (E-H). Dexmedetomidine (α 2-AR agonist) increased expression of IL-1, up to 2-fold (J). Dobutamine (β 1-AR agonist) increased expression of IL-6 (M), IL-1 β (N), and NGF (P) by

up to 50%. Terbutaline (β 2AR agonist) increased expression of IL-6 by 18-fold (Q), IL-1, up to 2-fold (R), and NGF by 50% (T).

Fig. 5. Effects of norepinephrine (NE) in stimulating phosphorylation of MAP kinases P38 (A), ERK (B), and JNK (C), as well as nuclear translocation of NFκB (D) in cultured rat keratinocytes, as measured by Western blots. NE (10^{-5} M) caused peak phosphorylation of all 3 MAP kinases at 5 min (A, B, and C). There was no NE effect on NFκB nuclear translocation (D).

Fig. 6.

Nociceptive and vascular changes after tibia fracture with and without systemic butoxamine (selective β 2AR antagonist, 2mg/kg, i.p.) treatment. Thirty minutes after injection butoxamine partially reversed the hindpaw mechanical allodynia (A) and the hindlimb unweighting (B) that developed after fracture, but didn't change hindpaw warmth (C) or edema (D). At the same dose, Butoxamine had no effect on mechanical nociceptive threshold in control animals (data not shown). * $P < 0.01$, ** $P < 0.001$ for FX-BTX, FX-Baseline or FX-Vehicle vs. control values, and # $p < 0.05$ for FX-BTX vs FX-Vehicle or FX-Baseline ($n = 8$ per group). FX: fracture, BTX: butoxamine.

Fig. 7.

Nociceptive and vascular changes after tibia fracture with and without systemic TB-2-081 (IL-6 receptor antagonist, 2mg/kg, s.c.) treatment. Treatment with TB-2-081 partially reversed the hindpaw allodynia (A), unweighting (B), and warmth (C) that developed after fracture, but had no effect on hindpaw thickness (D). At the same dose, TB-2-081 had no effect on mechanical nociceptive thresholds in control animals (data not shown). * $P < 0.01$, ** $P < 0.001$ for FX-Baseline ($n=15$), FX-Vehicle ($n=7$), or FX-TB-2-081 ($n=8$) vs. control values ($n=8$), and # $p < 0.01$, ## $P < 0.001$ for FX-TB-2-081 ($n=8$) vs. FX-Baseline ($n=15$), or FX-Vehicle ($n=7$) treatment. FX: fracture.

Fig. 8.

Activation of $\beta 2AR$ by terbutaline in control rats induced local mechanical allodynia and IL-6 production in keratinocytes. Top panels show intraplantar administration of terbutaline (5ug/30ul) caused hindpaw mechanical allodynia (A), and slight hindlimb unweighting (B) at 1 hr after injection in control rats. * $P < 0.05$, and ** $P < 0.01$ for terbutaline treatment vs. saline group or baseline values, # $P < 0.05$ and ## $p < 0.01$ for terbutaline treatment vs. saline treated group ($n=8$ for each group). Middle panels are changes in hindpaw skin cytokine and NGF protein levels in the plantar skin of control rats with and without terbutaline treatment. Cytokine production was measured by EIA 1 hr after intraplantar injection of vehicle or terbutaline. Intraplantar injection of terbutaline induced a significant increase in IL-6 (C)

cytokine level but did not change IL-1 β (D), TNF- α (E) or NGF (F) levels ($n = 7-8$ per cohort). ** $P < 0.01$ for terbutaline vs. vehicle controls. Bottom panels (G) are representative fluorescence photomicrographs of co-staining of IL-6 and keratinocytes (keratin label) in hindpaw skin sections from control rats, and from the ipsilateral hindpaw of control rats treated with intraplantar injected terbutaline. This study demonstrated that keratinocytes are the main source of IL-6 in responding to terbutaline in skin (Panel G, $n=3-4$ per cohort). Scale bar =20 μ m.

Fig. 9.

Proposed model for the integration of primary afferent, mast cell and sympathetic systems in supporting CRPS-related nociception. Previous reports describe the participation of primary afferent signaling and mast cell activation in supporting mediator production and, ultimately, nociceptor activation in this model. The results of our present experiments define a third β 2AR/IL-6-mediated sympathetic signaling pathway. Thus pain and sensitization in CRPS may be the result of several converging pathways. The balance of contributions from these pathways may explain clinical differences between patients both in terms of the manifestations of the syndrome as well as differences in sensitivities to treatment approaches.

Table 1

Primers used for real-time PCR

Gene	GenBank Accession #	Forward primer	Reverse primer	Product Size(bp)
ADRA1A	NM_017191	Acctcctcctcacctccact	gggtgacaatgggtggatag	204
ADRA1B	NM_016991	Agcggtagatgtcctgtgct	agatgaccgtggacaagacc	164
ADRA1D	NM_024483	Agcctctgcaccatctctgt	aaggagcacacggaagagaa	233
ADRA2A	NM_012739	Ggtaagggtggtgcgagat	cagcgcccttcttctatg	229
ADRA2B	NM_138505	Tgagccgagcattggagtacaact	aatgacagctgcaatgagccacac	89
ADRA2C	NM_138506	Tgggtttcctcatcggtttc	agtgccaggtagacaccaca	217
ADRB1	NM_012701	Gctctggacttcggtagacg	acttggggctgtttagcag	248
ADRB2	NM_012492	gagcacaagccctcaagac	tgggaaggcaatcctgaaac	209
ADRB3	NM_013108	cgcacctgggtctcattat	gaaggcagagttgcatagc	150
IL-1 β	NM_031512	agtctgcacagttccccaac	agacctgacttggcagagga	230
IL-6	NM_012589	ccggagaggagacttcacag	acagtgcacatcgctgttc	161
TNF- α	NM_012675	ctcccagaaaagcaagcaac	cgagcagggaatgagaagagg	210
NGF	XM_227525	acctcttcggacactctgga	gtccgtggctgtgtcttat	168
18S	X01117	cgcggttctattttgttgggt	agtcggcatcgtttatggtc	219