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# Immobilization Contributes to Exaggerated Neuropeptide Signaling, Inflammatory Changes, and Nociceptive Sensitization After Fracture in Rats

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**Abstract: A tibia fracture cast immobilized for 4 weeks can induce exaggerated substance P and calcitonin gene–related peptide signaling and neuropeptide-dependent nociceptive and inflammatory changes in the hind limbs of rats similar to those seen in complex regional pain syndrome (CRPS). Four weeks of hind limb cast immobilization can also induce nociceptive and vascular changes resembling CRPS. To test our hypothesis that immobilization alone could cause exaggerated neuropeptide signaling and inflammatory changes, we tested 5 cohorts of rats: 1) controls; 2) tibia fracture and hind limb casted; 3) hind limb casted, no fracture; 4) tibia fracture with intramedullary pinning, no cast; and 5) tibia fracture with intramedullary pinning and hind limb casting. After 4 weeks, the casts were removed and hind limb allodynia, unweighting, warmth, edema, sciatic nerve neuropeptide content, cutaneous and spinal cord inflammatory mediator levels, and spinal c-Fos activation were measured. After fracture with casting, there was allodynia, unweighting, warmth, edema, increased sciatic nerve substance P and calcitonin gene–related peptide, increased skin neurokinin 1 receptors and keratinocyte proliferation, increased inflammatory mediator expression in the hind paw skin (tumor necrosis factor-$\alpha$, interleukin [IL]-1$\beta$, IL-6, nerve growth factor) and cord (IL-1$\beta$, nerve growth factor), and increased spinal c-Fos activation. These same changes were observed after cast immobilization alone, except that spinal IL-1$\beta$ levels were not increased. Treating cast-only rats with a neurokinin 1 receptor antagonist inhibited development of nociceptive and inflammatory changes. Four weeks after fracture with pinning, all nociceptive and vascular changes had resolved and there were no increases in neuropeptide signaling or inflammatory mediator expression.**

***Perspective:** Collectively, these data indicate that immobilization alone increased neuropeptide signaling and caused nociceptive and inflammatory changes similar to those observed after tibia fracture and casting, and that early mobilization after fracture with pinning inhibited these changes. Early limb mobilization after fracture may prevent the development of CRPS.*

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Complex regional pain syndrome (CRPS) is characterized by allodynia, pain with movement, warmth, and edema in the injured extremity. The mechanisms mediating CRPS are unknown, but several observations suggest that limb immobilization plays a role. The traumatized limb is frequently immobilized in casts, splints, or fixators prior to the development of CRPS,[1,38] and CRPS patients tend to immobilize the limb to prevent movement-induced pain.[3,30] Furthermore, aggressively mobilizing the CRPS limb with physical therapy can alleviate symptoms.[29,30] Intriguingly, cast immobilization of the wrist for 4 weeks in normal

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experimental subjects causes skin warmth, hyperalgesia, and movement-evoked pain, symptoms partially mimicking CRPS.[42] Collectively, these data suggest that prolonged immobilization can contribute to the development of CRPS.

Population-based studies indicate that distal limb fracture is the most common cause of CRPS,[11,36] and we have developed a rat tibia fracture CRPS model. After tibia fracture with 4 weeks of cast immobilization, the rats developed chronic hind limb von Frey allodynia, warmth, increased protein extravasation, edema, and periarticular osteopenia, changes paralleling those observed in CRPS patients.[14] Surprisingly, control rats that had no fracture but were casted for 4 weeks also developed hind limb allodynia, warmth, increased protein extravasation, edema, and periarticular osteopenia, similar to the effects of tibia fracture with casting, but these changes resolved much more quickly in the cast-only rats.[14] Treatment with a substance P (SP) neurokinin 1 (NK1) receptor antagonist partially reversed the allodynia, warmth, spontaneous protein extravasation, and edema in both cast-only and tibia/casted rats.[14] These results suggest that SP signaling contributes to the CRPS-like changes observed with both cast immobilization and tibia fracture with casting.

The nociceptive and vascular changes characteristic of early CRPS suggest an inflammatory process, and there is extensive evidence that neurogenic inflammatory responses mediated by sensory afferent release of SP and calcitonin gene–related peptide (CGRP) are exaggerated in the CRPS limb[5,6,24,31,37,44] and in the rat fracture/cast CRPS model.[14,46] Furthermore, inflammatory cytokine levels are upregulated in CRPS skin blister fluid[19,20] and skin,[23] and levels of tumor necrosis factor-$\alpha$ (TNF-$\alpha$), interleukin-1$\beta$ (IL-1$\beta$), interleukin-6 (IL-6), and nerve growth factor (NGF) are elevated in the hind paw skin of fracture/casted rats.[34,35,47] Treating fracture/cast rats with a TNF inhibitor (etanercept), an IL-1 receptor antagonist (anakinra), an IL-6 receptor antagonist (TB-2-081), or an anti-NGF antibody (tanezumab) reduced allodynia and unweighting.[25,27,34,35] In addition, treating fracture/casted rats with an SP NK1 receptor antagonist prevented the upregulation of inflammatory mediators in the skin and reversed pain behavior.[45] These data suggest that facilitated SP signaling after fracture with casting caused increased inflammatory mediator expression in the fracture limb, resulting in nociceptive sensitization.

The aims of current study were to test the hypotheses that prolonged immobilization can facilitate neuropeptide signaling, causing inflammatory and nociceptive changes similar to those observed after tibia fracture with casting, and to determine whether early mobilization after fracture with intramedullary pinning can prevent or reverse those changes.

## 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, Brentwood, MO), which contains 1.0% calcium, .5% phosphorus, and 3.3 IU/g vitamin $D_3$, and were kept under standard conditions with a 12-hour light-dark cycle.

### Study Design

All cohorts of rats used in this study (other than the untreated controls) were enrolled in the same experimental protocol. First, all rats underwent right tibia fracture or hind limb cast immobilization, then 4 weeks later all casts were removed. The following day all rats underwent hind paw behavior testing (bilateral von Frey withdrawal thresholds, weight bearing, temperature, and thickness measurements), and then the rats were sacrificed and the hind paw skin, sciatic nerve, and lumbar spinal cord were collected for enzyme immunoassay (EIA), Western immunoblot, and immunohistochemical assays. The tibia fracture rats were divided into 3 different treatment cohorts at the time of fracture: 1) fracture stabilized by 4 weeks of cast immobilization, 2) fracture stabilized by intramedullary pinning, and 3) fracture stabilized by intramedullary pinning and 4 weeks of cast immobilization. One group of cast-only immobilized rats was injected with an SP NK1 receptor antagonist (LY303870, 20 mg/kg intraperitoneally [i.p.]) 4 hours before behavior testing.

### Drugs

The effects of an SP NK1 receptor antagonist (LY303870) were evaluated in hind limb cast immobilized rats. After 4 weeks of cast immobilization, the casts were removed and the next day LY303870 (20 mg/kg) was injected i.p. Approximately 4 hours later, hind paw von Frey thresholds, weight bearing, temperature, and thickness were determined, and at 8 hours after LY303870 injection the rats were euthanized and the hind paw skin collected for TNF-$\alpha$, IL-1$\beta$, IL-6, and NGF EIA. LY303870 has nanomolar affinity for the rat NK1 receptor; has no affinity for 65 other receptors and ion channels; has no sedative, cardiovascular, or core body temperature effects in rats at systemic doses up to 30 mg/kg; and is physiologically active for up to 24 hours after a single systemic dose of 10 mg/kg.[13,18,21]

### Surgery

Tibia fracture was performed under 2 to 4% isoflurane to maintain surgical anesthesia as we have previously described.[14,15] The right hind limb was wrapped in a stockinet (2.5 cm wide) and the distal tibia was fractured using pliers with an adjustable stop (Vise-grip; Petersen Manufacturing, Huntersville, NC) that had been modified with a 3-point jaw. The hind limb was