

Systemic Antiinflammatory Corticosteroid Reduces Mechanical Pain Behavior, Sympathetic Sprouting, and Elevation of Proinflammatory Cytokines in a Rat Model of Neuropathic Pain

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Background: Chronic pain models are commonly defined as either nerve-injury or inflammation models, but recent work suggests inflammatory processes are important in nerve injury-induced pain.

Methods: In the rat spinal nerve ligation model, the authors examined effects of systemic corticosteroid triamcinolone acetonide (TA) on the cytokine protein profile and sympathetic sprouting in the axotomized sensory ganglia, excitability of sensory neurons, and mechanical sensitivity.

Results: By postoperative day 3, marked increases (5- to 16-fold) in monocyte chemoattractant protein-1, growth-related oncogene (GRO/KC or CXCL1), and interleukin (IL)-6 were observed, whereas IL-4 and IL-2 levels fell more than fourfold. The increased cytokines and number of sympathetic basket formations in the sensory ganglia were reduced toward normal values by TA given starting at the time of injury. Interleukin-4 and IL-2 levels were not restored by TA. Systemic TA also reduced the firing rate and incidence of bursting activity, but not the overall incidence of spontaneous activity, in large- and medium-sized neurons. Mechanical hypersensitivity on postoperative day 3 was reduced by TA, and some effect could still be observed 4 days after cessation of TA. However, starting TA at day 7 was ineffective.

Conclusions: Several components of the spinal nerve injury model are responsive to corticosteroid, suggesting inflammatory processes are important in the development of neuropathic pain. The observation that TA was effective when given starting at the time of injury suggests that steroid treatment might alter the development of chronic pain after surgical procedures that involve nerve injury, such as amputation or hernia repair.

OUR understanding of chronic pain has benefited from a number of different animal models. Such models are often classified as either nerve injury or inflammation

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models (for review, see reference 1). In the peripheral nervous system, commonly used nerve injury models include complete or partial transection of the sciatic nerve or its branches,²⁻⁴ chronic constriction of the sciatic nerve,⁵ and spinal nerve ligation (SNL).⁶ Chronic inflammation models include those in which inflammatory substances such as carrageenan, Freund adjuvant, or zymosan are injected beneath the skin⁷ or deposited adjacent to a nerve.⁸

More recent studies have shown that the distinction between nerve injury and inflammation models is not clear-cut. Nerve injury, as a form of tissue damage, evokes an inflammatory response. This response not only mediates tissue repair and regeneration, but contributes importantly to chronic pain behaviors. For example, inflammatory cytokines and chemokines, as well as growth factors with similar functions, have been shown to be up-regulated in many nerve injury models, and genetic or pharmacologic reduction of these molecules can reduce pain behaviors and other pathologies present in these models. In addition, many studies have demonstrated important roles for resident and invading immune cells and for glial cells in nerve injury pain models. Neurons including nociceptors express functional receptors for many of these inflammatory mediators (for review, see references 9-12).

In view of the emerging importance of inflammatory effects on neurons, we recently developed a model in which effects of direct localized inflammation of the dorsal root ganglion (DRG) neurons could be examined in the absence of nerve injury.¹³ In this model, inflaming the L5 DRG by depositing a small drop of the immune stimulus zymosan in the nearby epidural space led to prolonged mechanical allodynia and hyperalgesia, increased spontaneous activity and hyperexcitability of the sensory neurons, and sprouting of sympathetic fibers around sensory neurons, which is implicated in neuropathic pain. We also measured increases in certain proinflammatory cytokines within the DRG, along with decreases in some antiinflammatory cytokines.

We were interested in examining the role of inflammation in the initiation of neuropathic pain by comparing the localized inflammation of the DRG model with a commonly used nerve injury model, the SNL model.⁶ In the SNL model, robust pain behaviors, sympathetic sprouting into the axotomized DRG, and spontaneous activity and enhanced excitability of the sensory neurons

are observed. The model involves ligation of the L5 (or L5 and L6) spinal nerves, allowing separation of the axotomized neurons in the L5-L6 DRG from the intact neurons in L4 that mediate the observed evoked pain behaviors. Although this is commonly used as a neuropathic pain model, like other nerve injury models,¹⁴ it has been shown to induce infiltration of macrophages and T cells into the axotomized and adjacent DRG.¹⁵ In this study, we report that the cytokine changes in the DRG induced by the SNL model bear many similarities to those observed in the localized inflammation model. This led us to test the response of the SNL model to subcutaneous injections of a commonly used corticosteroid antiinflammatory drug, triamcinolone acetonide (TA).

Materials and Methods

Animals

Sprague-Dawley rats obtained from Harlan (Indianapolis, IN) were housed in groups of two to four in 40 × 60 × 30-cm plastic cages with soft bedding under a 12–12 h day–night cycle. The rats were kept 7–10 days under these conditions before surgery and up to 2 weeks after surgery. The experimental protocol was approved by the Institutional Animal Care and Use Committees of the University of Cincinnati, Cincinnati, Ohio.

Procedures for Spinal Nerve Ligation and Triamcinolone Acetonide Injections

Male Sprague-Dawley rats weighing 200–250 g at the time of surgery were anesthetized with isoflurane. An incision was made on the back between L2 and S1. The L5 spinal nerve was exposed and tightly ligated with 6-0 silk and cut approximately 5 mm distal to the ligature. For experiments in which cytokine levels in the DRG were measured, both the L4 and the L5 spinal nerves were cut to increase the amount of tissue from DRGs with SNL obtained from each animal. The antiinflammatory corticosteroid TA was given beginning on the day of surgery, one subcutaneous injection on the back of the neck approximately 60 min before surgery (1.5 mg/kg), followed by one additional injection per day for the first 3 days after surgery, for a total of four injections. The control group received four injections of normal saline in the same volume. In other experiments, animals received the same series of injections but without the SNL procedure, or received the injections starting on postoperative day (POD) 7, as indicated. For sham surgeries used in cytokine measurement experiments, both of the L4–L5 spinal nerves were exposed, but without nerve ligation.

Behavioral Testing for Mechanical Allodynia

The testing procedure has been described in detail in previous publications.^{16,17} Rats were inspected and

tested every other day for 5 days before surgery (three testing sessions). After surgery, behavioral testing was performed on day 3 and day 7, and on additional days as indicated in the figures. To avoid potential bias, the person performing the behavioral testing was blinded as to the experimental group. Rats were placed in an acrylic glass box with a plastic mesh floor. To obtain percent withdrawal responses, von Frey filaments capable of exerting bending forces of 20, 40, 60, 80, and 120 mN, but each having the same tip diameter of 0.1 mm, were applied, in the order of ascending force, to six designated loci distributed over the plantar surface of the foot. Each filament was applied alternately to each foot and to each locus. The withdrawal threshold, defined as the force (filament) associated with 50% response of foot withdrawal, was calculated by fitting the force–response data to the Hill equation using a program, Microcal Origin 7.0 (Microcal Software, Inc., Northampton, MA).¹⁸ The mean withdrawal thresholds (baseline) of each hind paw before surgery were obtained from an average of three testing sessions (as day 0).

Immunohistochemical Staining for Sympathetic Fibers

Rats were anesthetized on POD 8 with pentobarbital sodium (40 mg/kg, intraperitoneal) and fixed by perfusing 200–300 ml Zamboni fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH = 7.4) through the left ventricle of the heart. The axotomized L5 DRG was removed, postfixed in the perfusion fixative for 1 h at 4°C, and embedded in gelatin overnight. The ganglia were horizontally sectioned with a Vibratome (Vibratome Company, St. Louis, MO) at a thickness of 40 μm.

Tissue sections were incubated in antibodies to tyrosine hydroxylase (TH) at a dilution of 1:1,000 for 48 h at 4°C, followed by reaction with biotinylated secondary antibody and, finally, with Vector ABC reagent (Vector Labs, Burlingame, CA). The TH antibody is an affinity purified polyclonal rabbit antibody; the antigen is purified denatured rat TH isolated from pheochromocytoma cells. Specificity is demonstrated by ability to stain the noradrenergic and dopamine systems in rat brain with low background (Pel-Freeze, Rogers, AR). Triton-X (0.3%) was used in all reaction solutions to enhance antibody penetration. Immunoreaction products were visualized by the diaminobenzidine method in the presence of H₂O₂ in 0.1 M phosphate buffer. Tissues were then mounted on gelatin-coated slides, air dried, dehydrated, and coverslipped for light-microscopic observation.

Slides from control and experimental groups were labeled with numbers so that the person performing the image analysis was blinded as to the experimental group.

Table 1. Cytokine Levels in the Axotomized DRGs on PODs 3 and 7

Cytokine	POD 3				POD 7			Estimated Detection Limit
	Normal (n = 5 Samples)	Sham (n = 3)	SNL (n = 3)	SNL + Steroid (n = 3)	Sham (n = 3)	SNL (n = 6)	SNL + Steroid (n = 4)	
GM-CSF	—	—	—	—	—	—	—	—
IL-1 α	14.0 \pm 2.4	9.3 \pm 1.7	11.2 \pm 6.1	2.8 \pm 2.0*	7.5 \pm 3.6	11.3 \pm 4.2	14.1 \pm 3.2	0.10 (0.25 pM)
MCP-1	21.8 \pm 2.9	30.6 \pm 8.5	106 \pm 52†	45.4 \pm 4.8†	28.8 \pm 12.1	63.2 \pm 20.1*	36.4 \pm 3.6*	0.06 (0.19 pM)
IL-4	1.8 \pm 0.5	2.2 \pm 1.9	0.1 \pm 0.1†	0.1 \pm 0.1†	1.8 \pm 1.0	0.0 \pm 0.0‡	0.4 \pm 0.1*	0.04 (0.12 pM)
IL-1 β	13.6 \pm 4.0	10.4 \pm 3.6	16.2 \pm 1.4	8.5 \pm 2.4	14.0 \pm 1.8	11.6 \pm 3.5	16.7 \pm 3.5	0.04 (0.09 pM)
IL-2	19.1 \pm 3.1	11.2 \pm 3.7	4.4 \pm 1.0†	3.7 \pm 0.7‡	12.9 \pm 4.1	4.7 \pm 1.1†	7.4 \pm 1.5†	0.10 (0.17 pM)
IL-6	11.0 \pm 1.9	25.4 \pm 11.1	180 \pm 37‡	106 \pm 10‡	11.3 \pm 1.8	42.2 \pm 4.0‡	59.4 \pm 10.0‡	0.16 (0.32 pM)
IL-10	3.8 \pm 2.6	5.0 \pm 3.2	1.3 \pm 1.3	0.7 \pm 0.6	1.3 \pm 1.3	1.0 \pm 0.8	4.1 \pm 1.9	0.06 (0.14 pM)
IL-12p70	5.3 \pm 1.3	3.3 \pm 0.7	1.6 \pm 0.6	1.2 \pm 0.7	3.3 \pm 1.4	1.6 \pm 0.3*	2.8 \pm 0.6	0.07 (0.04 pM)
IL-5	1.7 \pm 0.8	2.4 \pm 2.4	0.0 \pm 0.0	0.0 \pm 0.0	1.6 \pm 1.3	0.2 \pm 0.2	0.0 \pm 0.0	0.05 (0.15 pM)
IFN- γ	19.0 \pm 4.8	24.9 \pm 15.5	173 \pm 162	6.3 \pm 3.2	21.2 \pm 6.3	198 \pm 120	17.6 \pm 4.0	0.08 (0.22 pM)
IL-18	147 \pm 30	117 \pm 22	170 \pm 4	166 \pm 21	164.0 \pm 44.0	128 \pm 12	263 \pm 46	0.08 (0.14 pM)
GRO/KC	17.0 \pm 3.9	23.9 \pm 8.1	154 \pm 74†	31.8 \pm 0.8	22.2 \pm 5.9	28.1 \pm 8.4	39.1 \pm 15.1	0.03 (0.19 pM)
TNF- α	12.9 \pm 3.1	13.3 \pm 7.5	6.0 \pm 2.3	9.4 \pm 1.3	14.2 \pm 5.1	7.5 \pm 2.4	16.1 \pm 4.2	0.07 (0.18 pM)

Values are given as mean \pm SEM in units of pg/mg protein. Four steroid or normal saline (vehicle) injections were given daily starting immediately before the surgery. Significance of changes (vs. normal) is indicated by * ($P < 0.05$), † ($P < 0.01$), and ‡ ($P < 0.001$), based on ratio t tests (on the logs of the values, *i.e.*, examining the significance of the fold change). Each experimental measurement was on samples containing four ganglia combined from two rats, measured two or three times. The estimated detection limits were obtained from the lowest detectable cytokine concentration (as specified by the kit manufacturer) and the average experimental tissue average experimental protein weight, for each experiment.

DRG = dorsal root ganglion; GM-CSF = granulocyte-macrophage colony-stimulating factor; GRO/KC = growth-related oncogene; IFN = interferon; IL = interleukin; MCP = monocyte chemoattractant protein; POD = postoperative day; SNL = spinal nerve ligation; TNF = tumor necrosis factor.

In addition, all images were captured and analyzed by an investigator other than the one who performed immunohistostaining to avoid possible bias. Each DRG was sectioned into 15–20 sections that were then mounted on a single slide. Using ImagePro Plus software (Media Cybernetics, Inc., Silver Spring, MD), images from all sections of each DRG were captured under a light microscope (20 \times) equipped with a SPOT Insight colored digital camera (Diagnostic Instruments, Inc., Burlingame, CA) and stored in a personal computer for measurement. The number of neuronal somata surrounded by TH-immunoreactive basket-like structures or rings were counted from 4 (randomly chosen) out of 15–20 sections of the TH-immunostained DRGs. Only DRG neurons that were encircled by TH-immunoreactive fibers for at least two thirds of the circumference of the somata and that had a clearly visible nucleus were counted. The average density of the TH-immunoreactive basket/ring within each DRG was obtained by dividing the total number of baskets/rings by the size of the total measured cellular area (area in mm²).

Cytokine Measurement

Cytokine expression profiles in the DRG were evaluated on PODs 3 and 7 after the SNL (with or without TA treatment) or sham surgery using the Bio-Plex System (Bio-Rad, Hercules, CA) combined with the Linco 14-Plex Rat Cytokine Detection Kit (Linco Research, Inc., St. Charles, MO). A total of 14 rat cytokines were measured simultaneously from a single well according to the manufacturer's protocols, as previously described.¹³ Briefly, ipsilateral DRGs (L4 and L5) were isolated from

the rats and homogenized in lysis buffer (Bio-Rad) supplemented with a protease inhibitor cocktail (Sigma, St. Louis, MO) followed by centrifugation (13,000 rpm) at 4°C for 30 min to obtain extracted protein. Protein samples (25 μ l) extracted from DRG tissue were thawed and run in duplicate. A broad sensitivity range of standards (Linco Research, Inc.) ranging from 4.88 to 20,000 pg/ml was used to allow the quantization of a dynamic wide range of cytokine concentrations and provide the greatest sensitivity. This method was chosen because it has sensitivity and performance similar to enzyme-linked immunosorbent assay methods but requires much smaller sample volumes and is suitable for multiplexing.¹⁹

The concentrations of cytokines in these assays were calculated using a standard curve. A regression analysis was performed to derive an equation that was then used to predict the concentration of the unknown samples. The measured cytokines were interleukin (IL)-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-18, interferon γ , tumor necrosis factor α , granulocyte-macrophage colony-stimulating factor, growth-related oncogene (GRO/KC), and monocyte chemoattractant protein (MCP)-1.

The final concentrations of the 14 cytokines were obtained from an average of values observed in three to six samples for each time point and condition, as indicated in table 1. Each individual sample contained four DRGs combined from two rats, measured two or three times. Data were normalized to the amount of protein; similar results were obtained using normalization to the tissue weight.

Measurement of Spontaneous Activity and Excitability Parameters

At POD 7 (*i.e.*, 4 days after the last injection of steroid or saline), intracellular recording was performed on sensory neurons in whole DRG preparations isolated from saline- and steroid-treated rats. As described in previous publications,^{16,20} the ipsilateral L5 DRG was dissected out of the rat during barbiturate anesthesia, placed in the recording chamber, and mounted on the stage of an upright microscope (BX50-WI; Olympus America Inc., Center Valley, PA). A U-shaped stainless steel rod with three pieces of fine nylon filaments crossed from one side to the other was used to gently hold the ganglion in place within the recording chamber. The DRG was continuously perfused at a rate of 2–5 ml/min with oxygenated artificial cerebrospinal fluid containing 130 mM NaCl, 3.5 mM KCl, 1.25 mM NaH₂PO₄, 24 mM NaHCO₃, 10 mM dextrose, 1.2 mM MgCl₂, and 1.2 mM CaCl₂ (pH = 7.3). The temperature was maintained at 36° ± 1°C by a temperature controller.

Dorsal root ganglion cells were visualized under differential interference contrast. Intracellular, electrophysiologic recordings were made from each cell with a microelectrode filled with 2 M potassium acetate (pH = 7.2). Satisfactory recordings were obtained with electrodes of 50–80 MΩ from large and medium neurons, visually classified by the diameter of the soma (> 30 μm). The electrophysiologic data were collected with the use of single-electrode continuous current clamp (AxoClamp-2B; Axon Instruments, Inc., Union City, CA) and analyzed with pClamp 9 software (Axon Instruments, Inc.).

In experiments to determine the incidence of spontaneous activity, individual DRG neurons were first impaled with a recording electrode. If spontaneous activity was absent during the first 60 s of the impaling, incremental currents (up to 4 nA) were then injected to ensure that action potentials could be evoked indicating a healthy cell. If any spontaneous activity was present, we waited for 3 min to ensure that the activity was not caused by penetrating the somata with the sharp electrode. Next, the following parameters were measured using a series of current pulse injections, as described previously²¹: the threshold current (rheobase), action potential threshold, resting membrane potential, input resistance, and afterhyperpolarization of the recorded DRG cell. The resting potential was taken 3 min after a stable recording was first obtained.

Data Analysis

Significance was ascribed for $P < 0.05$. Behavioral time-course data were analyzed using two-way repeated-measures analysis of variance, with Bonferroni post test to determine on which days steroid-treated and saline-treated animals were significantly different, if an overall significant drug effect was observed. One-way analysis of

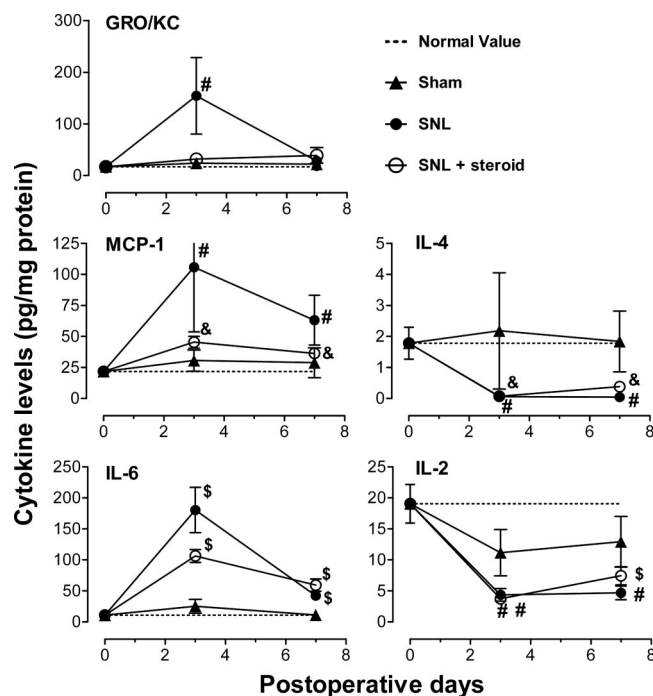


Fig. 1. Time course of cytokines significantly changed by spinal nerve ligation (SNL). The normal value in unoperated animals is indicated by the dotted line. Significance of changes (*vs.* normal) is indicated by & ($P < 0.05$), # ($P < 0.01$), and \$ ($P < 0.001$), based on ratio *t* tests (on the logs of the values, *i.e.*, examining the significance of the fold change). See also table 1. GRO/KC = growth-related oncogene; IL = interleukin; MCP = monocyte chemoattractant protein.

variance with Tukey multiple comparison tests was used to compare differences in TH basket density between control, SNL, and SNL + TA DRG sections. Significance of differences in average values of electrophysiologic parameters was determined using the Student *t* test, unless otherwise indicated (data not normally distributed were tested with the Mann-Whitney rank sum test). Significance of differences in proportions of bursting cells was determined using the Fisher exact test.

Results

SNL Increases Inflammatory Cytokines in the Axotomized DRG and Corticosteroid Treatment Partially Reverses These Increases

Levels of selected cytokines were measured in DRG from normal animals and from the experimental groups on PODs 3 and 7. The results are summarized in table 1 and figure 1. Of the 14 cytokines measured, all but one (granulocyte-macrophage colony-stimulating factor) were readily detectable in normal DRG. None were significantly elevated from normal levels in sham-operated animals on POD 3 or 7. Three cytokines were significantly elevated by SNL on day 3: MCP-1, GRO/KC (CXCL1), and IL-6, all of which are generally classified as proinflammatory cytokines. In each case, the cytokine level was reduced toward normal levels in animals

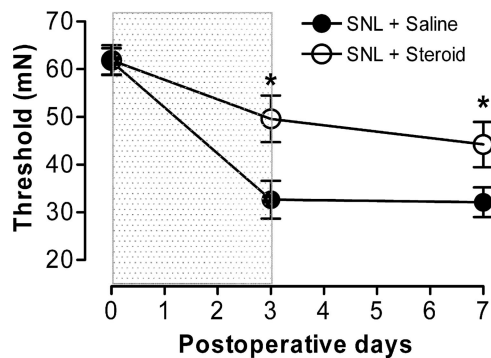


Fig. 2. Effect of systemic triamcinolone acetonide injection on mechanical pain behavior induced by spinal nerve ligation (SNL). Withdrawal threshold to mechanical stimulation of the hind paws was measured in the ipsilateral paw on the indicated postoperative day. The postoperative day 0 value is the average of three measurements taken over 5 days before the surgery. The shaded area indicates the time of triamcinolone acetonide injections. * Days on which there was a significant difference between steroid- and saline-treated animals (two-way repeated-measures analysis of variance).

treated with steroidal antiinflammatory TA ($1.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ for 4 days beginning just before the surgery), although the values in steroid-treated animals were (except for GRO/KC) still significantly higher than in normal animals. MCP-1 and IL-6 remained elevated in SNL animals on day 7, but the effects of steroid treatment were less apparent at this time. Two cytokines, IL-2 and IL-4, were significantly lower after SNL and remained so through POD 7. Interestingly, the steroid treatment did not seem to elevate these cytokines back toward normal levels. Similar results were obtained if the cytokines were normalized to tissue weight instead of to protein content. It should be noted that some of the average measurements for IL-4 (other than for the normal and day 7 sham groups) include values that were below the detection limit, limiting the quantitative comparisons between the experimental groups.

Corticosteroid Treatment Reduces Mechanical Hyperalgesia after SNL

As previously shown, SNL caused a marked mechanical hyperalgesia ipsilateral to the injury site which was evident by POD 3.⁶ Treatment of animals with TA just before surgery and once daily for the next 3 days caused a significant decrease in the mechanical pain behaviors evoked by SNL (overall P value for drug effect = 0.02; $n = 11$ TA- and 12 saline-treated SNL rats). This effect of the steroid was still evident on POD 7, *i.e.*, 4 days after the last steroid injection (fig. 2). In contrast, injecting the TA for a similar period of time, but starting on POD 7, after mechanical pain behaviors were well established, caused only a transient reduction of pain behavior which did not outlast the period of steroid injection ($n = 6$ TA- and 4 saline-treated SNL rats; fig. 3A). TA injection had no effect on pain behavior in normal, uninjured rats ($n = 4$; fig. 3B).

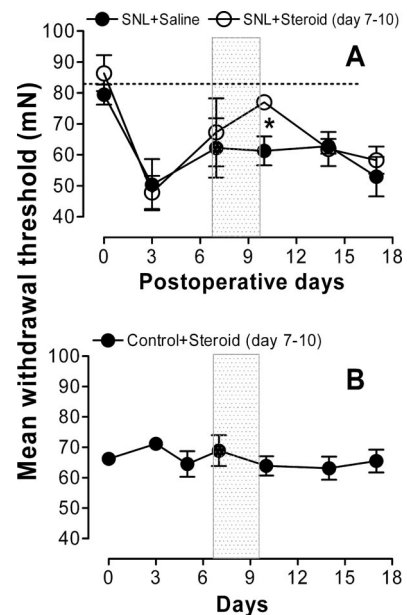


Fig. 3. Triamcinolone acetonide injection has only a transient effect if initiated after pain behaviors are established, and does not affect pain behavior in uninjured animals. (A) Effect of triamcinolone acetonide injection beginning on postoperative day 7, as indicated by the shaded area. * Only one measurement showed significant differences in individual t tests ($P = 0.004$ without correction for multiple testing). (B) Injection of steroid (shaded area) has no effect on cutaneous sensitivity in unoperated rats. SNL = spinal nerve ligation.

Corticosteroid Treatment Reduces Sympathetic Sprouting after SNL

After SNL, sprouting of sympathetic fibers particularly around large- and medium-diameter cells is revealed by immunostaining for TH. The fibers often form distinctive basket-like structures around the neurons. Examples of the basket formations in a saline-treated animal on POD 8 are shown in figure 4A, and examples from a TA-treated animal are shown in figure 4B. We quantified the degree of sympathetic sprouting by measuring the density of these basket structures in fixed sections of DRG on POD 8. In animals treated with TA ($n = 11$), just before and for the first 3 days after the SNL surgery, the density of such basket formations was reduced almost fourfold, though it was still significantly higher than in control animals ($n = 12$; fig. 5). As previously reported, basket formations were rare in normal, uninjured animals ($n = 8$).

Corticosteroid Treatment Reduces Incidence of Bursting in Spontaneously Active Neurons

Spinal nerve ligation is well known to cause an increase in excitability and spontaneous activity in the axotomized cells (see Discussion section). We examined spontaneous activity and several measures of excitability in large- and medium-diameter cells using intracellular recording from isolated whole DRG at 37°C on POD 7 ($n = 161$ cells from five steroid-treated animals and 137 cells from four saline-treated animals). Most of the excitability parameters measured were not significantly al-

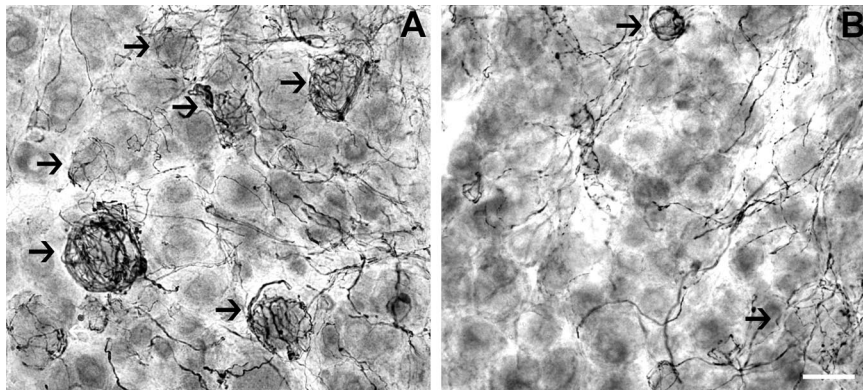


Fig. 4. Examples of sympathetic fiber basket formations in dorsal root ganglion tissue sections stained for tyrosine hydroxylase. Dorsal root ganglia were isolated on postoperative day 8. *Arrows* indicate examples of neurons surrounded by basket formations. (A) Example from a saline-injected spinal nerve-ligated animal. (B) Example from a triamcinolone acetonide-injected spinal nerve-ligated animal. Scale bar = 50 μm .

tered by steroid injection on PODs 0–4, including resting membrane potential (-60.0 ± 0.48 mV in cells from steroid-treated animals *vs.* -59.4 ± 0.62 mV in cells from saline-treated animals; $P = 0.42$), rheobase (0.85 ± 0.04 *vs.* 0.95 ± 0.05 nA; $P = 0.13$), action potential threshold (-41.6 ± 0.67 *vs.* -42.4 ± 0.83 mV; $P = 0.45$), action potential amplitude (31.2 ± 0.60 *vs.* 32.2 ± 0.63 mV; $P = 0.26$), and action potential duration (1.8 ± 0.05 ms in both groups; $P = 0.54$). However, the input resistance was significantly higher after steroid treatment (33.4 ± 1.41 *vs.* 29.1 ± 1.54 M Ω ; $P = 0.001$, Mann-Whitney test), and the duration of the afterhyperpolarization was significantly longer (3.5 ± 0.18 *vs.* 2.9 ± 0.16 ms; $P = 0.03$, Mann-Whitney test). As previously reported,²¹ we observed a relatively high incidence of spontaneous activity after SNL which could be readily classified as tonic, bursting, or irregular. The overall incidence of spontaneous activity did not differ between the steroid- and saline-treated animals (fig. 6A). However, a lower percentage of the spontaneously active cells showed a bursting pattern of activity after steroid treatment, when measured either as the overall incidence (fig. 6B) or as the percentage of spontaneously active cells that were of the bursting type (fig. 6C). In addition, the average firing frequency of those cells that

were spontaneously active was significantly lower after steroid treatment (fig. 6D).

Discussion

The primary findings of this study are that SNL causes robust increases in several proinflammatory cytokines within the DRG, and that a systemic antiinflammatory steroid, TA, given for 4 days beginning just before SNL, can significantly reduce behavioral pain for at least 7 days, reduce sympathetic sprouting, and mitigate increases in proinflammatory cytokines. These findings support the idea that the SNL nerve injury model has important inflammatory contributions. In addition, the observation that TA was effective when given starting at the time of injury suggests that steroid treatment might alter the development of chronic pain resulting from

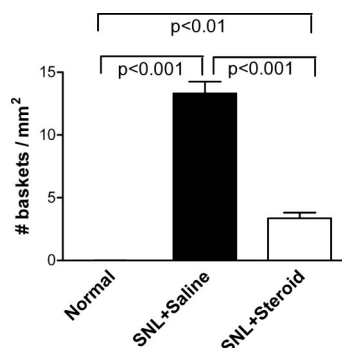


Fig. 5. Triamcinolone acetonide treatment for the first 3 days after spinal nerve ligation (SNL) markedly reduces the density of sympathetic fiber basket formations. Dorsal root ganglion sections were stained for tyrosine hydroxylase on postoperative day 8, and basket formations were counted as described in the Materials and Methods section. Indicated P values comparing each pair of experimental groups are from one-way analyses of variance with Tukey post test.

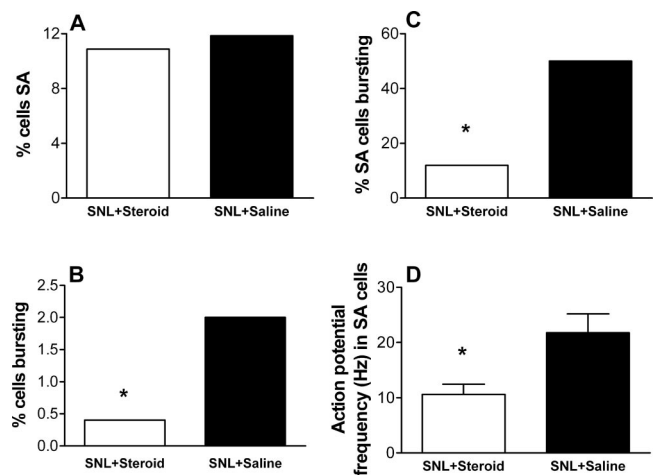


Fig. 6. Effect of steroid on spontaneous activity (SA). (A) Incidence of spontaneous activity in large and medium-diameter cells was not significantly altered by steroid treatment ($P = 0.85$, Fisher exact test). (B) The overall incidence of a bursting pattern of spontaneous activity was significantly lower after steroid treatment ($*P = 0.048$). (C) Incidence of bursting activity normalized to total number of spontaneously active cells was also significantly lower after steroid treatment ($*P = 0.03$). (D) The average action potential frequency in spontaneously active cells was significantly lower after steroid treatment ($*P = 0.006$, Student t test). SNL = spinal nerve ligation.

certain surgical procedures that involve nerve injury, such as amputation or hernia repair.

A number of studies have shown that glucocorticoids (applied systemically, locally, or intrathecally) can reduce pain behaviors in various rodent nerve injury models (e.g., references 22–24). These studies are generally similar to ours in demonstrating only a partial reduction of pain behavior. However, there are discrepancies in the literature about the ability of steroids to reduce pain behaviors if given after pain is well established. This procedure was ineffective in our study, but in some studies delayed application is effective (e.g., references 25 and 26). In addition, some studies found glucocorticoids to be effective in reducing thermal hyperalgesia (not examined in the present study) but not mechanical hyperalgesia,²⁶ or found that continuous drug application reduced hyperalgesia when daily injections did not.²⁷ An even more striking discrepancy comes from several studies showing that glucocorticoid receptor antagonists given intrathecally can reduce pain behaviors while glucocorticoids enhance them.^{28,29} These studies were initiated based on the finding that the glucocorticoid receptor in spinal cord neurons is up-regulated in the chronic constriction injury model. Glucocorticoids act through three primary mechanisms: direct regulation of transcription, inhibition of transcription effects mediated by nuclear factor κ B (an important transcription factor up-regulated in inflammation), and extragenomic effects. These mechanisms are thought to have different dose–response curves.³⁰ Hence, it is likely that understanding the conflicting results about effects of glucocorticoids in nerve injury models will require careful consideration of both dose–response and anatomical site of action of the drugs.

In our study, the primary effect on of TA on spontaneous electrical activity after SNL was to reduce the number of sensory neurons firing with a bursting pattern and to reduce the average firing frequency. Previous studies have documented the increase in spontaneous activity in large and medium diameter DRG neurons after SNL (e.g., references 31–34). The incidence of spontaneous activity observed in this study after SNL (11.1%) was not significantly different from that observed in our recent study using identical recording procedures (11.5% in large-diameter cells, 19.5% in medium-diameter cells).²¹ In contrast, the incidence of spontaneous activity in large and medium diameter cells is much lower in control rats, e.g., 5% in our previous study, with similarly low values observed in other studies using a variety of recording methods.^{17,35–38} In various models of chronic pain, spontaneous activity is one of the earliest abnormalities observed, and blocking this spontaneous activity is an effective way to block subsequent development of pain behaviors.³⁹ In the current study, we found that although TA was effective at reducing later-occurring events such mechanical pain, sympathetic sprouting,

and inflammatory cytokines, it had little effect on the overall incidence of spontaneous activity. This suggests that spontaneous activity may be “upstream” of these other, steroid-responsive effects, consistent with the fact that spontaneous activity is one of the earliest observed consequences of nerve injury. Another possibility is that the bursting form of spontaneous activity, which was the only type of spontaneous activity significantly reduced by TA, is much more effective than other forms of activity at inducing pain behaviors, sprouting, and/or cytokine production. Work in other systems indicates that the bursting form of activity may be particularly effective at releasing transmitters, and may specifically increase release of peptidergic transmitters in neurons that have peptidergic and nonpeptidergic cotransmitters.^{40,41}

Although we do not know why systemic steroid treatment reduces fiber sprouting, it may be explained by decreased expression of certain inflammatory cytokines such as IL-6, which has been reported to be able to cause sympathetic growth.^{42,43} Other cytokines such as GRO/KC may be involved, too, because GRO/KC is known to promote angiogenesis in various tissues,^{44,45} and sympathetic fibers are always associated with vascular processes in the DRG.^{46,47}

We recently examined the cytokine profile in the newly developed DRG localized inflammation model,¹³ which was designed to examine the effects of inflammation on neurons in the absence of injury (see introduction section). It is interesting that the cytokine profile bears many similarities to that reported in this study in the SNL model, a model designed to examine the effects of nerve injury. All the cytokines observed to increase on POD 3 in the SNL model were also observed to increase in the DRG inflammation model, with roughly comparable fold increases. Two additional cytokines, IL-18 and IL-1 β , were significantly elevated in the localized inflammation model but not the SNL model. However, these increases were much more modest than for other up-regulated cytokines in the localized inflammation of the DRG model (approximately 1.7 fold), so the nonsignificant (approximately 1.2-fold) increases we observed in SNL may reflect a similar trend, although not quite reaching significance. In both models, most cytokines that are elevated at POD 3 decline by POD 7. However, the two models diverge more at later times. Although both models still have significantly elevated MCP-1 levels on day 7, in the DRG inflammation model, the IL-18 level does not show the early peak typical of other elevated cytokines, being higher on day 7 than on day 3 and showing sustained elevation through at least day 14, and the IL-1 β increase, although modest, is also more sustained. As noted above, these cytokines are not significantly elevated in the SNL model and the measured values showed a tendency to decrease, not increase, by day 7. In the SNL model, it is IL-6 that shows a more sustained in-

crease, declining much more slowly than in the localized inflammation model.

The significant elevation of GRO/KC in both models suggests an important role for neutrophils, although it should be noted that cytokines may have roles in the nervous system that differ significantly from their immunologic roles in other tissues.⁴⁸ Neutrophil invasion of peripheral nerve has been demonstrated in several other models of nerve injury, where depletion of neutrophils attenuates pain behaviors.⁹ The SNL and DRG inflammation models also both show similar, sustained declines in IL-4, IL-2, and IL-12, although the IL-12 decrease was not significant in the SNL model until day 7. The decline in IL-4 is expected in an inflammatory setting, because this is generally considered an antiinflammatory cytokine. IL-2 and IL-12 are generally considered to be proinflammatory cytokines whose primary source is T cells; the observation that they decline rather than increase suggests that T-cell responses are not dominant in either model. An interesting finding in the current SNL study was that TA treatment was effective at reducing SNL-induced cytokine increases but had no ability to restore levels of the 3 cytokines that declined. This is in contrast to many examples from the immunology literature, in which IL-4 is increased by corticosteroid treatment, consistent with a general view that corticosteroids shift the immune system from a TH1 cell response toward a TH2 cell response.^{49,50} The failure of TA to increase IL-4 in this study also suggests that T-cell responses are not dominant in this model.

The finding that proinflammatory cytokines that increase on PDO3 have declined again by day 7, whereas the pain behaviors last much longer, suggests that these cytokines are more likely to play a role in initiation of the pain behaviors than in their maintenance. We also observed that TA treatment was much less effective in reducing pain behavior if initiated on POD 7, after pain behavior was well established. In this case, the TA effects did not outlast the application of the drug. This finding is in general agreement with previous work in that it implies that initiation and maintenance of chronic pain behaviors are distinct processes. For example, blockade of spontaneous activity at a nerve injury site is very effective at blocking development of pain behaviors if applied during the first week after the injury, but has only a temporary effect if initiated at a later time point, after pain behaviors are well established.³⁹

The overall gross similarity of the cytokine profiles in the SNL and localized inflammation models is perhaps not surprising, given that the SNL model involves an injury close to the DRG and has been previously shown to evoke immune cell infiltration and activation, which is more prolonged than the infiltration observed after the sciatic nerve is transected more distally, at the level of its trifurcation.¹⁵ The resulting pain behaviors are unlikely to be due entirely to immunogenic properties of the

sutures, rather than to nerve injury, because it has previously been shown that placing silk sutures adjacent to the DRG without ligating nerves will not evoke pain behaviors.⁵¹ This control was not included in the current study, however. Supporting the idea that the SNL model has a relatively large inflammatory component at the DRG level, due to the proximity of the nerve injury, we find that the CCI and axotomy models, in which the nerve injury is more distal, show much more modest and slower increases in some of the same cytokines. Our cytokine results highlight the difficulty in drawing a clean distinction between nerve injury and inflammatory models of pain, a point that has been made previously based on other lines of evidence (see introduction section). It seems likely that, at the level of the DRG, either type of model may expose the sensory neurons to increased levels of certain proinflammatory cytokines. Understanding the effects of these cytokines on the sensory neurons should contribute to understanding both nerve injury and inflammatory models of chronic pain.

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