Behavioral/Systems/Cognitive

Expression of Central Glucocorticoid Receptors after Peripheral Nerve Injury Contributes to Neuropathic Pain Behaviors in Rats

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Peripheral glucocorticoid receptors (GRs) play a significant role in the anti-inflammatory effects of glucocorticoids; however, the role of central GRs in nociceptive behaviors after peripheral nerve injury (neuropathic pain behaviors) remains unknown. Here we show that the development of neuropathic pain behaviors (thermal hyperalgesia and mechanical allodynia) induced by chronic constriction nerve injury (CCI) in rats was attenuated by either the GR antagonist RU38486 ($4 = 2 > 1 = 0.5$ μg) or a GR antisense oligonucleotide administered intrathecally twice daily for postoperative days 1–6. The development of thermal hyperalgesia and mechanical allodynia after CCI also was prevented in adrenalectomized rats, whereas the GR agonist dexamethasone (100 μg/kg) given subcutaneously twice daily for postoperative day 1–6 restored CCI-induced neuropathic pain behaviors in the adrenalectomized rats. Mechanistically, CCI induced a time-dependent and region-specific expression of neuronal GRs primarily within the spinal cord dorsal horn ipsilateral to nerve injury, which showed a time course parallel to that of the development of neuropathic pain behaviors. Moreover, the expression of neuronal GR after CCI was mediated in part through an elevated spinal level of interleukin-6 (IL-6) and protein kinase Cγ (PKCγ), because intrathecal treatment with an IL-6 antiserum, a PKC inhibitor (cheryrithrine), or PKCγ knock-out substantially reduced the expression of neuronal GRs as well as neuropathic pain behaviors after CCI. These findings indicate a central role of neuronal GRs in the mechanisms of neuropathic pain behaviors in rats and suggest a potential role for GR antagonists in clinical management of neuropathic pain.

Key words: glucocorticoid; spinal; neuropathic pain; NMDA receptor; interleukins; protein kinase C

Introduction

Pain after injury to the nervous system (neuropathic pain) is a major chronic pain condition that remains difficult to treat. Both peripheral and central mechanisms of neuropathic pain have been proposed (Wall et al. 1974; Devor, 1983, Dougherty and Willis, 1991; Dubner, 1991; Yamamoto and Yaksh, 1992; Mao et al., 1995; Woolf and Mannion, 1999). Peripheral glucocorticoid receptors (GRs) play a significant role in the anti-inflammatory effects of glucocorticoids, which are mediated mainly through interactions between GRs and intracellular elements such as activating protein-1 at the site of tissue inflammation (Neeck et al., 2002); however, GRs have also been located in spinal cord dorsal horn neurons (De Nicola et al., 1989; Cintra et al., 1993). Activation of neuronal GRs contributes to central changes related to neuronal injury (Cameron and Dutia, 1999) and the process of learning and memory (Quirarte et al., 1997; Oitzl et al., 1998; Roosendaal et al., 1999, 2003). Of interest is that neuronal degeneration induced by activation of the NMDA receptors

(NMDARs) in the rat magnocellular nucleus basalis was enhanced in the presence of GR agonists (Abraham et al., 2000), and conversely, hippocampal neuronal apoptosis resulting from a glucocorticoid challenge was mediated through NMDARs and metabotropic glutamate receptors (Lu et al., 2003), suggesting a functional interaction between neuronal GRs and NMDARs. Indeed, the GR level increases in the hippocampus after a focal brain injury (McCullers et al., 2002) and in the spinal cord after traumatic injury (Yan et al., 1999). Moreover, glucocorticoids could have neurotoxic effects contributing to neuronal damage (Fuxe et al., 1996). To date, a central role of GRs in the mechanisms of neuropathic pain has not been examined.

Proinflammatory cytokines including interleukin (IL)-1β, IL-6, IL-10, and tumor necrosis factor-α are produced at both peripheral and central loci in response to peripheral nerve injury (Deleo et al., 1996; Arruda et al., 1998; Murphy et al., 1999; Okamoto et al., 2001; Raghavendra et al., 2002; Schafers et al., 2003). These cytokines play a significant role in inflammatory responses in part through intracellular mediators such as protein kinase C (PKC) and cAMP (Barkhudaryan and Dunn, 1999). Moreover, cytokines have been suggested to play a role in the development of neuropathic pain (Opree and Kress, 2000; Sweitzer et al., 2001; Milligan et al., 2003; Schafers et al., 2003; Watkins and Maier, 2003), and interleukins have been shown to enhance conditioned fear memory in part through the effects of glucocorticoids (Song

et al., 2003). Thus, it is possible that cytokines and intracellular PKC may induce changes in neuronal GRs in response to peripheral nerve injury.

In a rat model of neuropathic pain induced by chronic constriction nerve injury (CCI) (Bennett and Xie, 1988), we examined the hypothesis that central GR would contribute to the mechanisms of neuropathic pain behaviors after peripheral nerve injury, in part through the expression of neuronal GRs mediated by proinflammatory interleukins and intracellular PKC.

Materials and Methods

Animal models of peripheral nerve injury. Adult male Sprague Dawley rats weighing 275–325 gm (Charles River Laboratories, Wilmington, MA) were used. The animal room was artificially lighted from 7:00 A.M. until 7:00 P.M. The experimental protocol was approved by our Institutional Animal Care and Use Committee. CCI rats were produced by loosely ligating a common sciatic nerve according to the method of Bennett and Xie (1988). Another rat model of peripheral never injury in which the L5 spinal nerve root on one side was tightly ligated according to the method of Kim and Chung (1992) was used in some experiments to confirm the findings in CCI rats. Sham rats were made following the same surgical procedure except for nerve ligation. Adrenalectomized rats (250–275 gm) were purchased from Charles River Laboratories. Adrenalectomized rats were given *ad libitum* access to food and 0.9% NaCl solution instead of water and used for the experiments at 7 d after the adrenal surgery that removed bilateral adrenal glands, including the medullary component. PKCγ knock-out (KO) mice and the matched wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Intrathecal catheterization and drug delivery. An intrathecal catheter was implanted in a rat under the same surgical conditions, and a PE10 catheter was inserted onto the level of the lumbar enlargement (~8.5 cm from the incision site for this rat age group) according to the method described previously (Yaksh and Rudy, 1976; Mao et al., 2002). Those rats exhibiting postoperative neurological deficits (e.g., paralysis) or poor grooming were excluded from the experiments as described previously (Mao et al., 2002). The following drugs were purchased from Sigma (St. Louis, MO): RU38486, spironolactone, cheryrithrine, dexamethasone, and IL-6. RU38486, cheryrithrine, dexamethasone, or spironolactone was dissolved in 10% ethanol solution. IL-6 was dissolved in normal saline. IL-6 antiserum was purchased from R & D Systems (Minneapolis, MN) and dissolved in PBS. All drugs were injected intrathecally in a 10 μl volume followed by a 10 μl saline flush.

For those experiments using GR oligonucleotides, antisense sequences overlapping the respective initiation codon were designed for targeting GR mRNA: GR antisense (TGG AGT CCA TT GCA AAT), GR sense (ATT TGC AAA TGG ACT CCA), and the mixed base (TGA AGT TCA GTG TCA ACT). Sequencing was performed by MWG-Biotech (High Point, NC). Oligonucleotides were dissolved in 0.9% saline and injected (5 μM, i.t.) twice a day. After each experiment, tissue samples were harvested to examine neurochemical changes.

Behavioral tests and statistical analysis. Animals were habituated to the test environment daily (a 60 min session) for 2 d before baseline testing. The testing procedure for thermal hyperalgesia was performed according to a previously published method (Hargreaves et al., 1988). Temperature was set to have the baseline latency at ~10–12 sec and a cutoff of 20 sec. Mechanical allodynia was examined by applying a von Frey filament to the plantar surface of each hindpaw (Tal and Bennett, 1994). The cutoff force was 20 gm. All testing was conducted between 9:00 A.M. and 1 P.M. Data from both thermal hyperalgesia (withdrawal latency in seconds) and mechanical allodynia (threshold bending force in grams) tests were analyzed by using repeated measure two-way ANOVA across testing time points to detect overall differences among treatment groups. Whenever applicable, the data were also examined by using repeated measure two-way ANOVA across treatment groups to examine overall differences among testing time points. In both cases, when significant main effects were observed, the *post hoc* Newman–Keuls tests were performed to determine sources of differences. Differences were considered to be statistically significant at the level of $\alpha = 0.05$.

ELISA. Plasma samples were taken between 10:00 A.M. and noon to minimize the variation of the plasma cortisol level. The cortisol ELISA kit (catalog #RE 520 61) was purchased from IBL Immuno-Biological Laboratories (Minneapolis, MN). The standard curve was generated using the reference standard set supplied in the kit. The samples were measured according to the instructions accompanying the kit. Hydrocortisone (Sigma) was used as control, and different levels were included in every assay. The results were read using a microplate reader (Bio-TeK Instruments Winooski, VT) at wavelengths of 450 and 620 nm. The plasma cortisol concentration was calculated based on the standard curve and presented in nanograms per milliliter.

Immunocytochemical staining. Rats were anesthetized with pentobarbital and transcardially perfused with 200 ml of normal saline followed by 200–300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The lumbar spinal cords were dissected, postfixed for 1.5 hr, and kept overnight in 30% sucrose in 0.1 M PB. Tissues from both experimental and control groups were then mounted together in OCT compound and frozen on dry ice. The spinal cords (20 μm sections) were cut on a cryostat, mounted serially onto microscope slides, and stored at −80°C. Immunocytochemical staining was used to detect GRs (1:1000, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), neuronal-specific nuclear protein (NeuN) (1:500, mouse monoclonal; Chemicon, Temecula, CA), PKCγ (1:500, mouse monoclonal; Santa Cruz Biotechnology), and IL-6 (1:200, goat polyclonal; R & D Systems). Lumbar spinal cord sections were blocked with 1% goat serum in 0.3% Triton for 1 hr at room temperature and incubated overnight at 4°C with a primary antibody. For controls, a primary antibody was omitted and antigen absorption (for GRs) was used in control sections. The sections were then incubated for 1 hr at room temperature with a corresponding FITC- or Cy3-conjugated secondary antibody (1:300; Chemicon). For double staining, a second primary antibody was added after the incubation with the first primary antibody for the same procedure as described above. Four to six nonadjacent spinal sections were randomly selected, analyzed using a fluorescence microscope (Olympus), recorded using a digital camera, and processed using Adobe Photoshop.

Western blot. Rats were decapitated rapidly (<1 min) under anesthesia, and the lumbar spinal cord segments and corresponding dorsal root ganglia (DRG) were removed. The spinal segments were separated into the ipsilateral and contralateral side as well as the dorsal and ventral part and homogenized in SDS sample buffer containing a mixture of proteinase inhibitors (Sigma). The lumbar segments were harvested because CCI has the major impact at these spinal segments, and the intrathecal drug delivery also was aimed at this site. Protein samples were separated on SDS-PAGE gel (4–15% gradient gel; Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride filters (Millipore, Bedford, MA). The filters were blocked with 3% milk and incubated overnight at 4°C with a primary antibody [GR: rabbit polyclonal, 1:1000; PKA: 1:1000, rabbit polyclonal (both from Santa Cruz Biotechnology); PKCγ: mouse monoclonal, 1:400 (Zymed Lab, San Francisco, CA); IL-6: goat polyclonal, 1:1000 (R & D Systems); IL-10: goat polyclonal, 1:1000 (Santa Cruz Biotechnology) and 1 hr at room temperature with HRP-conjugated secondary antibody (1:700; Amersham Biosciences, Arlington Heights, IL).

The blots were visualized in ECL solution (NEN, Boston, MA) for 1 min and exposed onto hyperfilms (Amersham Biosciences) for 1–10 min. The blots were then incubated in a stripping buffer (67.5 mM Tris, pH 6.8, 2% SDS, and 0.7% β-mercaptoethanol) for 30 min at 50°C and reprobed with a polyclonal rabbit anti-β-actin antibody (1:20,000; Alpha Diagnostic International, San Antonio, TX) or in some cases with both β-actin and extracellular signal-related kinase 2 (ERK2) (1:2000, rabbit polyclonal; Cell Signaling Technology, Beverly, MA) as loading controls. The Western analysis was made in triplicates. The density of specific bands was measured with a computer-assisted imaging analysis system and normalized against loading controls. Differences were compared using repeated measure one-way ANOVA followed by *post hoc* Newman–Keuls tests.

RT-PCR. Total RNA was isolated from the lumbar spinal cord segments, divided into the dorsal and ventral horn and ipsilateral and contralateral side, by using TRIZOL Reagent (TEL-TEST, Friendswood,

TX). After incubation for 15 min at 4°C, chloroform was added for the phase separation. The upper aqueous phase was collected, and RNA was precipitated through mixing the sample with isopropyl alcohol. The RNA pellet was washed once with 75% ethanol and air-dried, which was finally redissolved in RNase-free water. $A260/A280$ ratios were between 1.6 and 1.8.

RT-PCR was performed using the Titan One Tube RT-PCR System (Roche, Indianapolis, IN). Each PCR amplification with a volume of 25 μl contained 100 ng of total RNA, 0.4 μM each primer, 0.2 mM each deoxynucleotide, 5 mM dithiothreitol, 5 U RNase inhibitor, 1.5 mM $MgCl_2$, and 0.5 μl of enzyme mix. The reactions were performed in an MJ research thermocycler using individual programs as follows: GR: forward primer (AAA TCT TTG TTG GGC TCA GGC TTC CTT GT), reverse primer (GAA GAG AAA CGA GCA AGC ATA G), program (95°C for 30 sec, 43°C for 1 min, 72°C for 1 min); mineralocorticoid receptor (MR): forward primer (TAT CTG CAC TGT CGC TCT ATT ACT CTA CC), reverse primer (AAA TCT TTG TTG GGC TCA GGC TTC CTT GT), program (95°C for 30 sec, 50°C for 1 min, 72°C for 1 min); IL-6: forward primer (CGA AAG TCA ACT CCA TCT GCC), reverse primer (GGC AAC TGG CGT GAA GTC TCT), program (95°C for 1 min, 55°C for 1 min, 72°C for 1 min); PKCγ: forward primer (GCT CCC ACA TCA GAT GAG ATC CA), reverse primer (CGT CCT GGG CTG GCA CCG AAG AA), program (95°C for 1 min, 55°C for 2 min, 72°C for 3 min); PKCα: forward primer (ACC CTC AGT GGA ATG AGT CCT TCA CGT), reverse primer (TTA GAT GGC TGC TTC CTG TCT TCT GAA), program (95°C for 1 min, 55°C for 2 min, 72°C for 3 min). There were 32–35 cycles in all reactions except for PKCα, in which 40 cycles were used to improve the yield. Each program ended with 7 min at 68°C and stored at 4°C. Every PCR was accompanied by one negative control reaction without template RNA. PCR products were analyzed by gel electrophoresis on an ethidium bromide-stained 1% agarose gel (Sigma) in Tris–borate–EDTA buffer. The amount of RNA per RT-PCR sample was normalized using PCR with primers specific for β-actin: forward primer (TAC AAC CTC CTT GCA CC), reverse primer (ACA ATG CCG TGT TCA ATG G), program (95°C for 1 min, 55°C for 1 min, 72°C for 1 min). Each band was then measured with a computer-assisted imaging analysis system and normalized against the loading control. Differences were compared using a one-way ANOVA followed by *post hoc* Newman–Keuls tests.
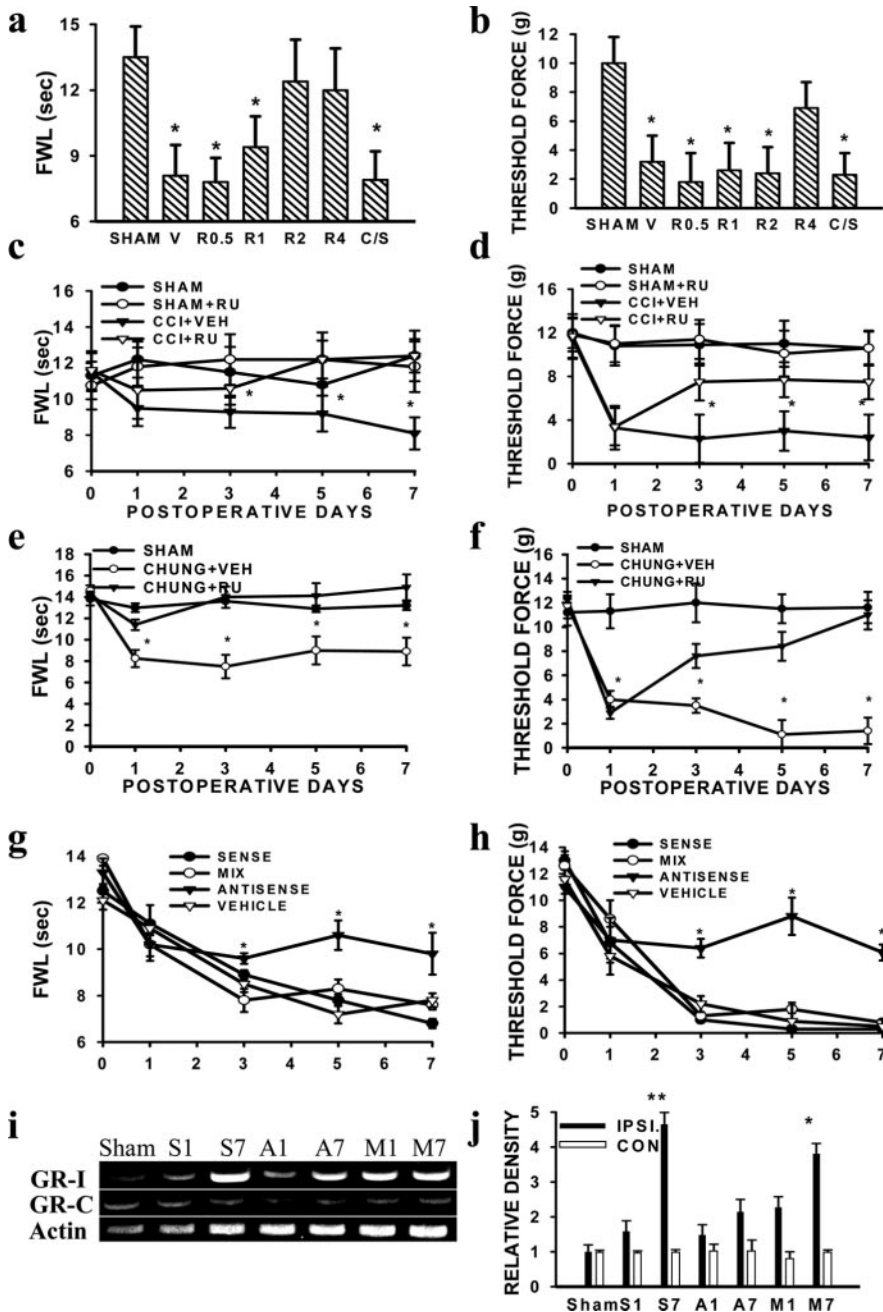

Figure 1. *a*, *b*, The dose–response effects of RU38486 on thermal hyperalgesia (*a*) and mechanical allodynia (*b*) examined on postoperative day 7 in CCI rats. The mineralocorticoid receptor antagonist spironolactone [3 μg; CC1 plus spironolactone (C/S)] did not affect hyperalgesia and allodynia in CCI rats. V, CCI+vehicle; R0.5 to R4, CCI rats treated with 0.5–4 μg RU38486. *c–f*, The development of thermal hyperalgesia (*c*, *e*) and mechanical allodynia (*d*, *f*) in CCI rats (*c*, *d*) or Chung rats (*e*, *f*) was diminished by the GR antagonist RU38486 (2 μg; CCI+RU or CHUNG+RU), but not vehicle (CCI+VEH or CHUNG+VEH), given twice daily for the first 6 postoperative days. RU38486 alone did not affect the behavioral responses in sham rats (*c*, *d*, sham+RU). In *a–f*, *p < 0.05 compared with sham rats. *g*, *h*, The development of thermal hyperalgesia (*g*) and mechanical allodynia (*h*) in CCI rats was diminished in CCI rats treated with the GR antisense OND (5 μM, i.t.) twice daily for 6 consecutive postoperative days. *p < 0.05 compared with each of the rest of the groups. *i*, *j*, The GR expression (RT-PCR) was substantially attenuated in CCI rats treated with the GR antisense OND. *p < 0.05 and **p < 0.01 compared with day 1 of each corresponding group. S1, S7, A1, A7, and M1, M7, Day 1 and day 7 from CCI rats treated with the GR sense (S), antisense (A), and a mixed base (M), respectively. In all figures, refer to the main text for the number of animals per group.

Results

Role of central GRs in neuropathic pain behaviors

The results from a series of behavioral experiments, using the GR antagonist RU38486, the GR agonist dexamethasone, GR oligonucleotides (ONDs), or adrenalectomy (ADL), collectively indicate that central GRs contributed to the development of neuropathic pain behaviors after peripheral nerve injury.

Effect of the GR antagonist RU38486

The effects of the GR antagonist RU38486 on neuropathic pain behaviors were examined in eight groups of rats including (1) CCI plus vehicle, (2–5) CCI plus 0.5, 1, 2, and 4 μg of RU38486, (6) CCI plus the

MR antagonist spironolactone (3 µg) [the selected spironolactone dose was used to block MR in a previous study examining the role of MR in morphine-induced locomotion (Marinelli et al., 1998)], (7) sham plus RU38486, and (8) sham plus vehicle. Each agent was given twice daily (intrathecally) for 6 postoperative days beginning immediately after operation. RU38486, but not spironolactone, dose-dependently ($4 = 2 > 1 = 0.5$ µg) reduced thermal hyperalgesia and mechanical allodynia as compared with vehicle-treated CCI rats (Fig. 1a–d) ($p < 0.05$; $n = 6$–9). RU38486 alone did not change thermal or mechan­ical nociceptive threshold in sham-operated rats (Fig. 1c,d) ($p > 0.05$; $n = 5$). Repeated treatment with RU38486 (2 µg, i.t.) using the same regimen for CCI rats also attenuated the development of thermal hyperalgesia and mechanical allodynia in the Chung model (Fig. 1e,f) ($p < 0.05$; $n = 6$), suggesting a broad effect of RU38486 on neuropathic pain behaviors after peripheral nerve injury.

Effect of GR antisense ONDs
Although there are reports on mice with genetically altered GRs, the mice are either lethal or unavailable to us (Tronche et al., 1999; Gartner et al., 2002; St. Hilaire et al., 2003). As an alternative, an antisense OND against GRs was delivered to the lumbar spinal segments via an intrathecal catheter. In this set of experiments, CCI rats each received a GR antisense OND (5 µM), sense OND, mixed base OND, or a vehicle. Each treatment was given twice daily for the first 6 postoperative days, and the spinal cord samples were harvested. The development of thermal hyperalgesia and mechanical allodynia was significantly attenuated in those CCI rats treated with the GR antisense as compared with the remaining groups (Fig. 1g,h) ($p < 0.05$; $n = 5$–7). The same antisense OND treatment did not affect the baseline noci­ceptive responses in sham rats ($p > 0.05$; $n = 5$). The effects of the GR antisense OND on neuropathic pain behaviors were associated with a substantial reduction of the GR expression (RT-PCR) in CCI rats treated with the GR antisense OND as compared with other OND treatment groups (Fig. 1i,j) ($p < 0.05$; $n = 5$–7).

Effect of ADL
The development of neuropathic pain behaviors after CCI was also examined in ADL rats lacking the ability to produce corticoids (Smith et al. 1991; Castonguay et al., 2002). The baseline plasma cortisol level was barely detectable by ELISA in ADL rats, and CCI did not result in an increase in the plasma cortisol level in ADL rats (Fig. 2a) ($p > 0.05$; $n = 6$). In contrast, there was a time-dependent change in the plasma cortisol level in CCI rats without ADL (Fig. 2a) ($p < 0.05$; $n = 6$). In non-ADL rats, the plasma cortisol level was dramatically increased 1 d after opera
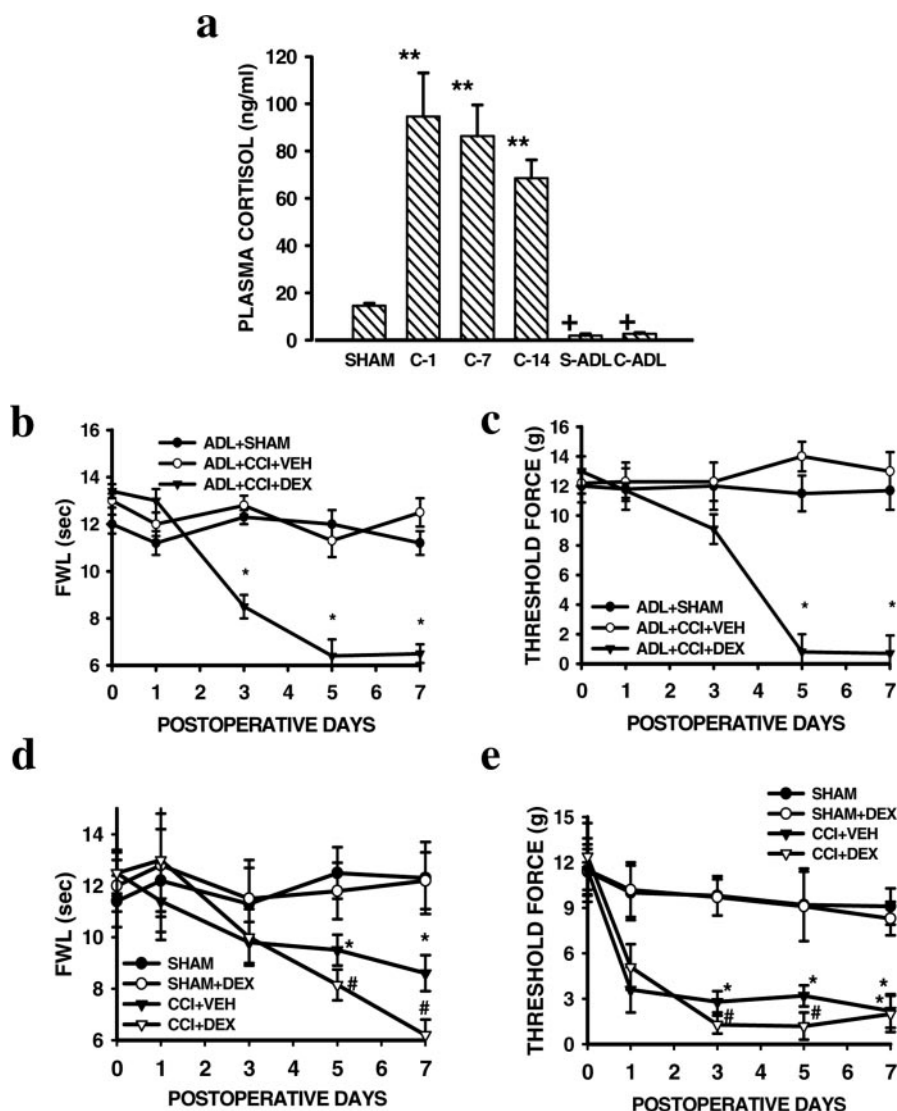

Figure 2. *a*, Changes in the plasma cortisol level after CCI. C-1 to C-14, Day 1 to day 14 after CCI; S-ADL and C-ADL, sham and CCI rats with adrenalectomy, respectively. **$p < 0.01$ compared with sham rats. $^+p < 0.05$ compared with both sham and CCI rats. *b, c*, ADL rats failed to develop thermal hyperalgesia (*b*) or mechanical allodynia (*c*) after CCI (ADL+CCI+VEH) compared with ADL rats with sham operation (ADL+SHAM), which was restored in ADL rats treated subcutaneously with twice daily 100 µg/kg dexamethasone (ADL+CCI+DEX) for postoperative days 1–6. *d, e*, Dexamethasone (DEX; 4 µg) given intrathecally twice daily for the first 6 postoperative days exacerbated the development of thermal hyperalgesia (*d*) and mechanical allodynia (*e*) in CCI rats. Dexamethasone alone did not affect the behavioral responses in sham rats (SHAM+DEX). *$p < 0.05$ compared with sham rats. #$p < 0.05$ compared with both sham and CCI+VEH groups.

tion, reflecting an acute stress response, and remained elevated up to at least 14 d after CCI as compared with sham operation (Fig. 2a) ($p < 0.05$; $n = 6$). ADL did not change baseline thermal or mechanical nociceptive threshold, nor did it change grooming and survival as compared with non-ADL rats.

After CCI, ADL rats failed to develop neuropathic pain behaviors as compared with sham operation (Fig. 2b,c) ($p > 0.05$; $n = 5$–6), whereas non-ADL rats developed significant neuropathic pain behaviors during the same period (Fig. 1c,d). The development of neuropathic pain behaviors after CCI was restored in ADL rats, however, when treated with dexamethasone (100 µg/kg, s.c.) twice daily for postoperative days 1–6 (Fig. 2b,c) ($p < 0.05$; $n = 7$), whereas the same dexamethasone treatment did not affect the baseline nociceptive responses. A similar dexamethasone regimen has been shown to restore the glucocorticoid effect on the insulin-mediated muscle protein synthesis in ADL rats (Long et al., 2003).
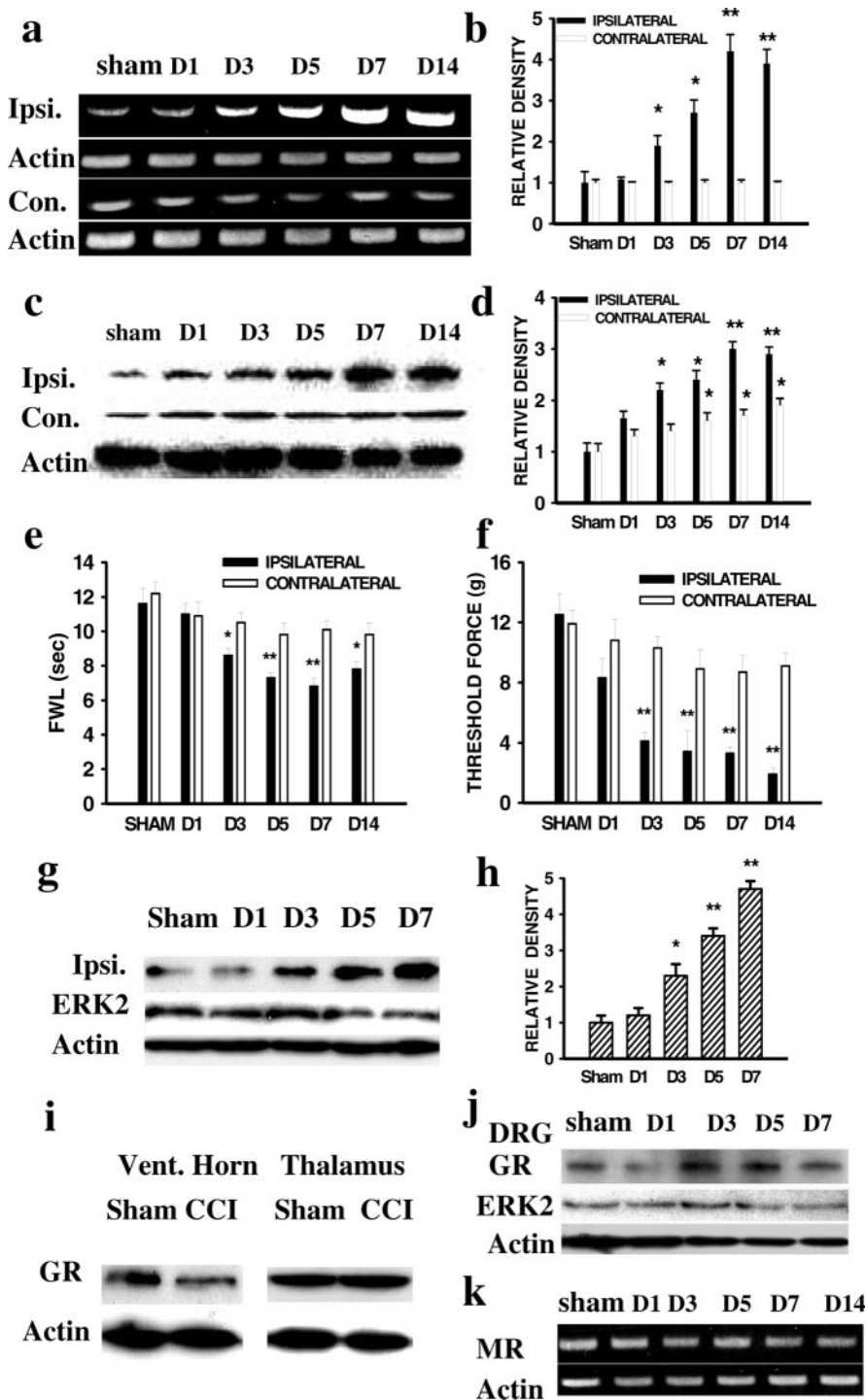
Figure 3. *a, c,* Time course of the GR expression (*a,* RT-PCR; *c,* Western blot, 95 kDa) within the spinal cord dorsal horn ipsilateral (Ipsi.) and contralateral (Con.) to CCI or sham operation (sham). Actin (β-actin, 42 kDa) is a loading control. *b, d,* Statistical analysis of the GR expression. Relative density, Relative density of RT-PCR and Western blot bands calculated by normalizing each band with its corresponding loading control band. *e, f,* Time course of the development of thermal hyperalgesia (*e*) and mechanical allodynia (*f*) after CCI. FWL, Foot-withdrawal latency. *g, h,* Time course of the GR expression (Western blot) and statistical analysis within the spinal cord dorsal horn ipsilateral to L5 nerve root ligation (the Chung model). Both β-actin and ERK2 are loading controls. In all panels, D1–D14, postoperative day 1 to day 14; *$p < 0.05$ and **$p < 0.01$ compared with sham rats on the same side of the spinal cord dorsal horn. *i–k,* The GR expression was not significantly changed after CCI in the ipsilateral spinal cord ventral horn (*i*), contralateral thalamus (*i*), or lumbar DRG (*j*) when examined on day 7 after CCI, nor was there a significant change in MR expression within the ipsilateral spinal cord dorsal horn after CCI (*k,* RT-PCR).

Effect of the GR agonist dexamethasone
To examine whether dexamethasone would exacerbate the development of neuropathic pain behaviors in non-ADL rats, the synthetic GR agonist dexamethasone (2 or 4 μg) or a vehicle was administered intrathecally twice daily for the first 6 postoperative days to both CCI and sham rats. CCI rats treated with dexamethasone developed significant thermal hyperalgesia and mechanical allodynia that followed a time course similar to that of CCI rats treated with a vehicle, indicating that dexamethasone did not shorten the onset of neuropathic pain behaviors. Both thermal hyperalgesia and mechanical allodynia were exacerbated, however, in CCI rats treated with 4 μg (not 2 μg) of dexamethasone (Fig. 2*d,e*) ($p < 0.05$; $n = 5–6$). Dexamethasone (4 μg) alone did not affect thermal and mechanical nociceptive threshold in sham rats (Fig. 2*d,e*) ($p > 0.05$; $n = 5–6$).

Expression of neuronal GRs after CCI
To explore the possible mechanisms of GR-mediated neuropathic pain behaviors in CCI rats, we first examined whether CCI would alter the expression of central GRs. CCI but not sham operation produced a time-dependent GR expression within the spinal cord dorsal horn ipsilateral to the site of nerve injury as revealed by both RT-PCR and Western blot (Fig. 3*a–d*) ($n = 4–6$). The significant increase in both GR mRNA and protein level was detected on postoperative day 3 in CCI rats and lasted at least up to postoperative day 14 (Fig. 3*a–d*) ($p < 0.05$). Although there was a moderate increase in the GR expression (Western blot) on the contralateral spinal cord dorsal horn in CCI rats as compared with sham operation, the GR expression was significantly greater on the side ipsilateral rather than contralateral to CCI (Fig. 3*b,d*) ($p < 0.05$). For controls, a set of samples from sham rats was taken on days 1, 3, 5, and 7 ($n = 3–4$). In addition, spinal cord samples were also taken from a group of naive rats ($n = 4$). Because there were no differences in the spinal GR expression (RT-PCR and Western blot) in sham rats across these time points as compared with the naive rats ($p > 0.05$) and the GR expression reached a stable level in CCI rats on postoperative day 7 (Fig. 3*a–d*), the data from day 7 after sham operation were presented (Fig. 3*a–d*).

The time course of GR changes was parallel to that of the development of thermal hyperalgesia and mechanical allodynia in CCI rats shown both in previous studies (Bennett and Xie, 1988; Mao et al.,

1992a; Yamamoto and Yaksh, 1992) and in the present study (Fig. 3e,f) (n = 5–6). A similar pattern of GR expression within the ipsilateral spinal cord dorsal horn was confirmed in the Chung model induced by ligating the L5 lumbar spinal nerve root (Fig. 3g,h). In contrast, there were no significant changes in the GR expression from the ipsilateral lumbar DRG, the spinal cord ventral horn, and the contralateral thalamus (Fig. 3i,j), nor was there a change in the MR level within the ipsilateral spinal cord dorsal horn after CCI (Fig. 3k).

The topographic distribution of GR immunoreactivity (GR-ir) also indicated a predominant increase in GR-ir within the spinal cord dorsal horn ipsilateral to CCI. GR-ir was increased primarily within superficial laminas (I–II), as compared with deeper laminas (III–VI), of the ipsilateral spinal cord dorsal horn (Fig. 4A,B). The majority of GR-expressing cell profiles expressed NeuN-ir (a neuronal marker), indicating a major association of GRs with neuronal cells (Fig. 4C–E). Of interest is that a number of GR-expressing cell profiles expressed PKCγ-ir, and almost all PKCγ-expressing cell profiles within laminas I–II also showed GR-ir (Fig. 4F–H). Thus, the data from RT-PCR, Western blot, and immunohistochemistry indicate that peripheral nerve injury induced a time-dependent and region-specific increase in neuronal GR expression within the spinal cord dorsal horn.

Role of IL-6 in GR expression

Several interleukins are proinflammatory cytokines that contribute to inflammatory responses after tissue injury (Barkhudaryan and Dunn, 1999). Our immunohistochemical data showed an increased level of IL-6-ir within the superficial laminas of the ipsilateral spinal cord dorsal horn in CCI rats (Fig. 4I,J), the same spinal locus with an increased GR expression

after CCI. This initial finding, along with the data indicating IL-6-induced GR upregulation in human osteoblast-like cell lines (Dovio et al., 2001), led us to examine whether IL-6 served as a mediator between peripheral nerve injury and the expression of neuronal GR. Indeed, CCI induced an increase in IL-6 (RT-PCR and Western blot) with a time course similar to that of the GR expression (Fig. 5a–d), whereas the change in IL-10 was minimal as compared with IL-6 after CCI (Fig. 5e) (n = 5). Changes in IL-6 mRNA from spinal cord dorsal horn samples indicate a *de novo* production of IL-6 in the CNS in response to peripheral nerve injury.

To further examine the relationship between IL-6 and the GR expression, an IL-6 antiserum (5 μg) or control serum was given intrathecally once a day for postoperative days 1–6 beginning immediately after the operation. The IL-6 antiserum dose was chosen based on the literature (Milligan et al., 2003; Schafers et al., 2003) and our pilot experiment. IL-6 antiserum, but not con-
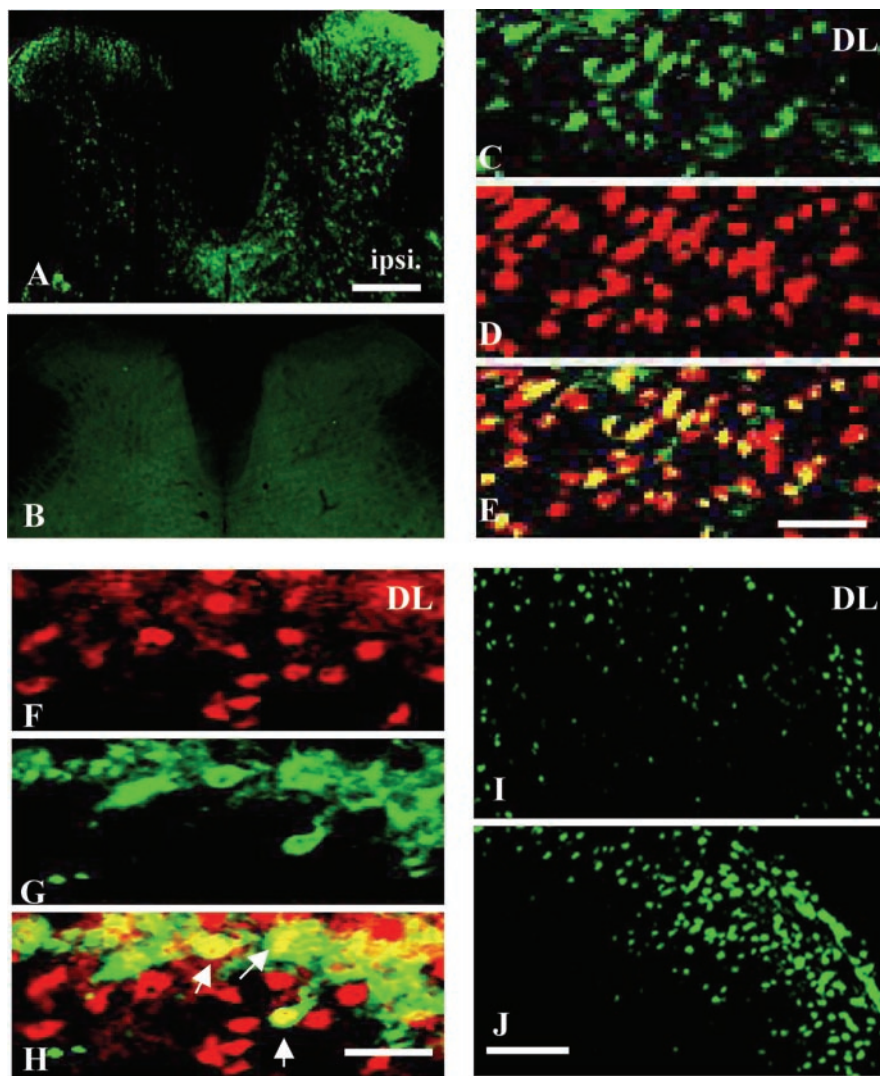

Figure 4. a, b, Topographic distribution of GR in a spinal cord section (a) taken from a representative CCI rat on postoperative day 7, showing differences in the GR expression within the spinal cord dorsal horn ipsilateral (ipsi.) and contralateral to CCI. b, Control section with the GR antigen absorption. c–h, Colocalization (e or h) of GR (c) and NeuN (d) or GR (f) and PKCγ (g) within the ipsilateral spinal cord dorsal horn of a representative CCI rat on postoperative day 7. h, Arrows indicate coexpression of GR and PKCγ. i, j, Topographic distribution of IL-6 within the ipsilateral spinal cord dorsal horn of a representative sham (i) and CCI (j) rat on postoperative day 7. Scale bars: a, b, 120 μm; i, j, 100 μm; c–e, 60 μm; f–h, 40 μm. DL, Dorsolateral part of the spinal cord dorsal horn indicating the orientation of spinal sections shown in c–e, f–h, i, j.

trol serum, significantly attenuated the GR expression (Fig. 5f) (p < 0.05; n = 5–6) and neuropathic pain behaviors (Fig. 5g,h) (p < 0.05; n = 5–6) in CCI rats when examined on postoperative day 7.

In addition, exogenous IL-6 itself (33 μg, i.t.) given once daily for 6 d to naive rats without nerve injury induced a significant increase in the GR expression (RT-PCR and Western blot) within the spinal cord dorsal horn as compared with vehicle-treated naive controls (Fig. 6a,b) (p < 0.05; n = 4–5). The IL-6-induced GR expression returned to the baseline level at 7 d after the discontinuation of the IL-6 treatment in these naive rats (Fig. 6b) (p > 0.05). The IL-6 treatment that induced the GR expression also resulted in thermal hyperalgesia and mechanical allodynia in these naive rats, when examined on day 7 after the IL-6 treatment for 6 d. Both thermal hyperalgesia and mechanical allodynia were attenuated at 30 min after the intrathecal administration of the GR antagonist RU38486 (2 μg) (Fig. 6c,d) (p < 0.05; n = 5–8).
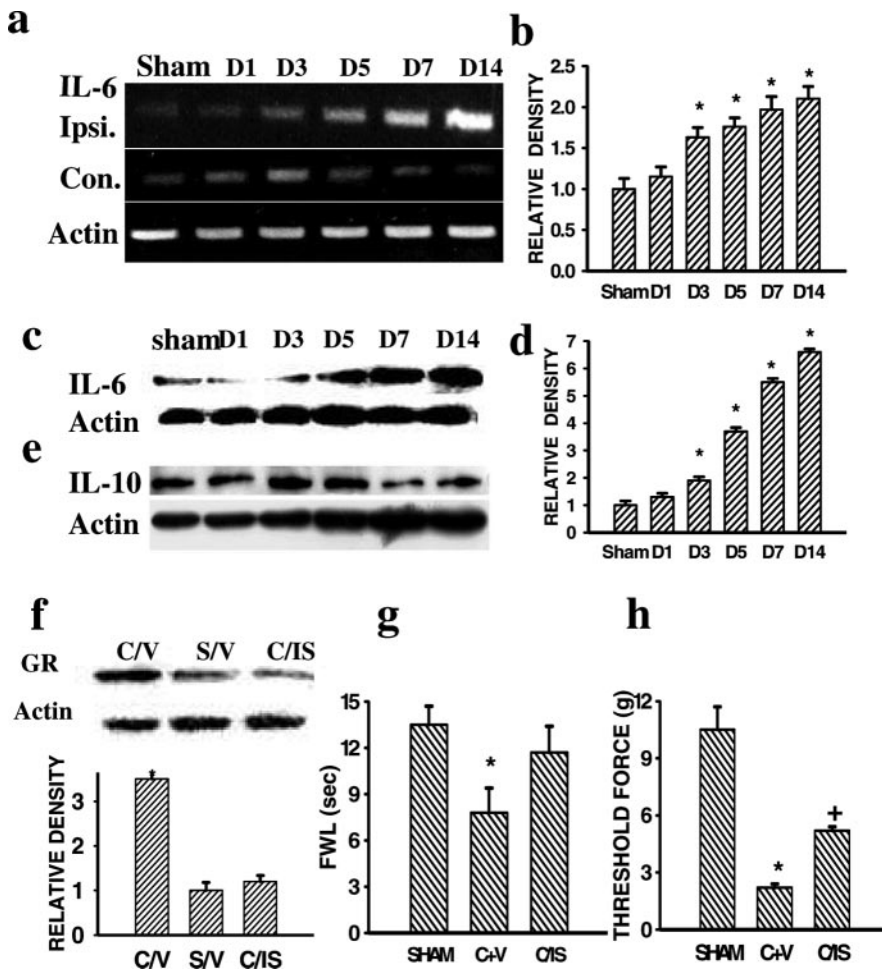
Figure 5. *a, b,* RT-PCR (*a*), Western blot (*c*) (27 kDa), and statistical analysis (*b, d*) showing the time course of changes in IL-6 within the spinal cord dorsal horn of sham and CCI rats. *$p < 0.05$ compared with sham rats (ipsilateral side only). *e,* There were no significant changes in IL-10 within the ipsilateral spinal cord dorsal horn after CCI. *f,* IL-6 antiserum (5 μg, i.t.) given once daily for the first 6 postoperative days attenuated the GR expression within the ipsilateral spinal cord dorsal horn of CCI rats. C/V, CCI plus control serum; S/V, sham plus control serum; C/IS, CCI plus IL-6 antiserum. *$p < 0.05$ compared with CCI rats treated with IL-6 antiserum. *g, h,* IL-6 antiserum (5 μg, i.t.) given once daily for the first 6 postoperative days attenuated the development of thermal hyperalgesia (*g*) and mechanical allodynia (*h*) in CCI rats when examined on day 7. C+V, CCI plus control serum; SHAM, sham plus control serum; C/IS, CCI plus IL-6 antiserum. *$p < 0.05$ compared with sham rats. +$p < 0.05$ compared with both sham rats and CCI rats treated with control serum.

RU38486 alone did not affect the baseline withdrawal latencies to thermal or mechanical stimulation ($p > 0.05$) ($n = 4$). Together with the results from IL-6 antiserum in CCI rats, the data indicate that IL-6 contributed to the GR expression after CCI.

Role of PKCγ in GR expression
Previous studies have suggested that PKC and cAMP/PKA may be potential intracellular mediators of the IL-6 effects (Barkhudaryan and Dunn, 1999). Thus, we asked whether PKC or PKA could mediate GR expression by first examining changes of both PKC (PKCγ and PKCα) and PKA after CCI. CCI induced a significant upregulation of PKCγ, but not PKCα, within the spinal cord dorsal horn with a time course similar to that of the GR expression (Fig. 7a–d) ($p < 0.05$; $n = 5$). In contrast, there was no significant change in the PKA expression after CCI as compared with sham operation (Fig. 7d) ($p > 0.05$; $n = 5$). These findings are consistent with a known role of PKCγ in neuropathic pain behaviors (Mao et al., 1995; Malmberg et al., 1997).

If PKCγ played a role in the IL-6-induced GR expression after

CCI, IL-6 antiserum should prevent the upregulation of both GRs and PKCγ in CCI rats. In those CCI rats treated with IL-6 antiserum (5 μg, i.t.) given once a day for postoperative days 1–6, the PKCγ upregulation was significantly reduced when examined on day 7 as compared with those given a control serum (Fig. 7e) ($p < 0.05$; $n = 5$). Conversely, exogenous IL-6 itself (33 μg, i.t.) given once daily for 6 d to naive rats without nerve injury induced a significant increase in PKCγ within the spinal cord dorsal horn as compared with vehicle-treated naive controls (Fig. 7f,g) ($p < 0.05$; $n = 4$–5). The PKCγ expression induced by IL-6 returned to the baseline in these naive rats at 7 d after the discontinuation of the IL-6 treatment (Fig. 7g) ($p > 0.05$; $n = 4$).

Moreover, the GR expression was significantly attenuated in those CCI rats treated with the selective PKC inhibitor cheryrithrine (10 μg, i.t.), but not a vehicle or a low dose cheryrithrine (2.5 μg), given twice a day for 6 postoperative days (Fig. 8a) ($p < 0.05$; $n = 5$). Cheryrithrine (10 μg, i.t.) also effectively attenuated the GR expression in naive rats treated with IL-6 (33 μg, i.t.) given once daily for 6 d as compared with the IL-6 alone group (Fig. 8a) ($p < 0.05$; $n = 4$–5), whereas cheryrithrine (10 μg) alone did not affect the GR expression in sham rats (Fig. 8a) ($p > 0.05$, $n = 4$).

The involvement of PKCγ in the GR expression after CCI was further indicated from the experiment using PKCγ KO and matched WT mice. Although the GR expression was upregulated in the WT mice 7 d after CCI, this CCI-induced GR expression was nearly abolished in PKCγ KO mice as compared with the WT mice (Fig. 8b) ($p < 0.05$; $n = 5$). There was a lack of baseline PKCγ expression in uninjured PKCγ KO mice as compared with uninjured PKCγ WT mice (Fig. 8c). Neuropathic pain behavior (thermal hyperalgesia) after CCI was significantly attenuated in PKCγ KO mice as compared with PKCγ WT mice in the present experiment (Fig. 8d) ($p < 0.05$; $n = 5$), a finding similar to that shown in a previous report using another peripheral nerve injury model (Malmberg et al., 1997). Collectively, the data from (1) CCI rats treated with cheryrithrine, (2) naive rats treated with IL-6 and cheryrithrine, and (3) CCI mice lacking the PKCγ expression indicate that PKCγ is an important intracellular mediator between the effect of IL-6 and GR expression after CCI.

Discussion
The present study demonstrates that (1) peripheral nerve injury induced a time-dependent expression of neuronal GRs primarily within the ipsilateral spinal cord dorsal horn, (2) the expression of neuronal GRs was mediated, at least in part, through an elevated spinal level of IL-6 and PKCγ, and (3) spinal neuronal GRs contributed to the development of neuropathic pain behaviors

after CCI. These findings indicate that GRs play a central role in contributing to the cellular mechanisms of neuropathic pain behaviors in rats.

Our data suggest a possible sequence of events related to the expression of neuronal GRs in CCI rats. Peripheral nerve injury elevated cytokines such as IL-6 in the CNS, as indicated by an increased spinal level of both IL-6 mRNA and protein, leading to the expression of neuronal GRs through intracellular mediators such as PKCγ. A critical site involved in this process is the spinal cord dorsal horn ipsilateral to nerve injury, because the spinal cord ventral horn, the DRG, and the contralateral thalamus showed no significant changes in the GR expression after CCI. Several lines of evidence support a role of IL-6 and PKCγ in the expression of neuronal GRs. (1) Both IL-6 and PKCγ were increased after CCI with a time course parallel to that of the GR change. (2) The expression of neuronal GRs was prevented by either blocking IL-6 with an antiserum or inhibiting PKC by the PKC inhibitor cheryrithrine; the same dose of cheryrithrine also blocked the upregulation of the spinal cannabinoid-1 receptor after CCI in our previous study (Lim et al., 2003). (3) The GR expression was substantially diminished in PKCγ KO mice, and PKCγ is known to be involved in the mechanisms of neuropathic pain behaviors in rodents (Mao et al., 1995; Malmberg et al., 1997). These findings, however, do not exclude the possibility that additional proinflammatory cytokines and intracellular signaling pathways could be activated by nerve injury (Opree and Kress, 2000; Sweitzer et al., 2001; Milligan et al., 2003) and contribute to the expression of neuronal GRs.

Neuronal GRs can be activated by circulatory corticosteroids and/or locally produced neurosteroids known to interact with GRs (Compagnone and Mellon, 2000; Plassart-Schiess and Baulieu, 2001; Vallee et al., 2001). Our data showed a significant and sustained increase in the plasma cortisol level after nerve injury, suggesting that an altered corticosteroid level could serve as an important source of endogenous GR agonists after CCI. Notably, the time course of GR expression generally parallels that of behavioral changes in CCI rats. Although there was a moderate change in the GR expression within the spinal cord dorsal horn contralateral to CCI as compared with sham operation, statistically significant behavioral changes were not observed on the contralateral hindpaw, a finding that is similar to a number of neurochemical changes observed in this and other animal models of neuropathic pain (Mao et al., 1995).

Our data demonstrate a role of GRs in the development of neuropathic pain behaviors after CCI. Collectively, the development of neuropathic pain behaviors in CCI rats was attenuated through the following: (1) intervention of those cellular elements (e.g., IL-6, PKCγ) mediating the expression of GRs; (2) inhibition of GRs with the GR antagonist RU38486 (RU38486 also attenuated the development of neuropathic pain behaviors in the
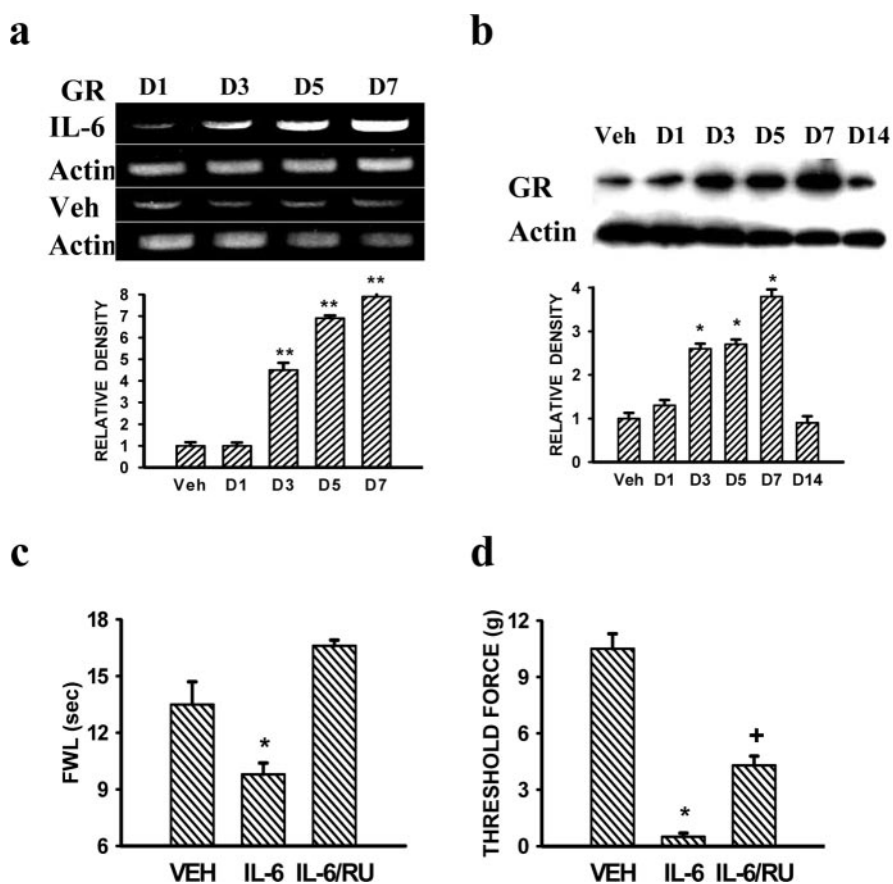

Figure 6. *a, b,* RT-PCR (*a*) and Western blot (*b*) and statistical analysis showing the time course of GR expression within the spinal cord dorsal horn of naive rats induced by IL-6 (33 μg, i.t.) given once daily for 6 consecutive days. D1–D7, Day 1 to day 7 of the IL-6 treatment. Note that the GR expression returned to baseline in *b* at 7 d after the discontinuation of IL-6 treatment (D14). *$p < 0.05$ compared with vehicle (veh). *c, d,* A single injection of the GR antagonist RU38486 (RU; 2 μg, i.t.) on day 7 attenuated both thermal hyperalgesia (*c*) and mechanical allodynia (*d*) induced by IL-6 (33 μg, i.t.) given once daily for 6 consecutive days in naive rats. *$p < 0.05$ compared with vehicle (VEH).

Chung model of nerve injury); (3) prevention of an increase in endogenous GR agonists after CCI in ADL rats; and (4) genetic alteration of the GR expression with a GR antisense OND. Conversely, neuropathic pain behaviors were exacerbated in CCI rats treated with the synthetic GR agonist dexamethasone, and a daily supplement with subcutaneous dexamethasone restored CCI-induced neuropathic pain behaviors in ADL rats. Several technical considerations are worth mentioning. First, although RU38486 has been used extensively as a conventional GR antagonist and is the only one available commercially, RU38486 does have other actions such as those of an antiprogesterone (Mao et al., 1992b). The antiprogesterone effect is unlikely to be contributory in CCI rats, because the experiment using GR antisense ONDs yielded results similar to those using RU38486 in exclusively male rats. Second, given that MR was not increased after CCI and the MR antagonist spironolactone did not change neuropathic pain behaviors, MR also is unlikely to be involved in this process. Third, intrathecal drug injection may potentially influence DRG and regions beyond the targeted spinal segments. A primary site of RU38486 action should be within the spinal cord dorsal horn in the present experiments, however, because (1) the intrathecal injection technique has been shown to be a method of regional drug delivery (Mao et al., 1992a; Yamamoto and Yaksh, 1992) and the agent was delivered in a small volume (10 μl) through a catheter threaded to the level of lumbar spinal seg-
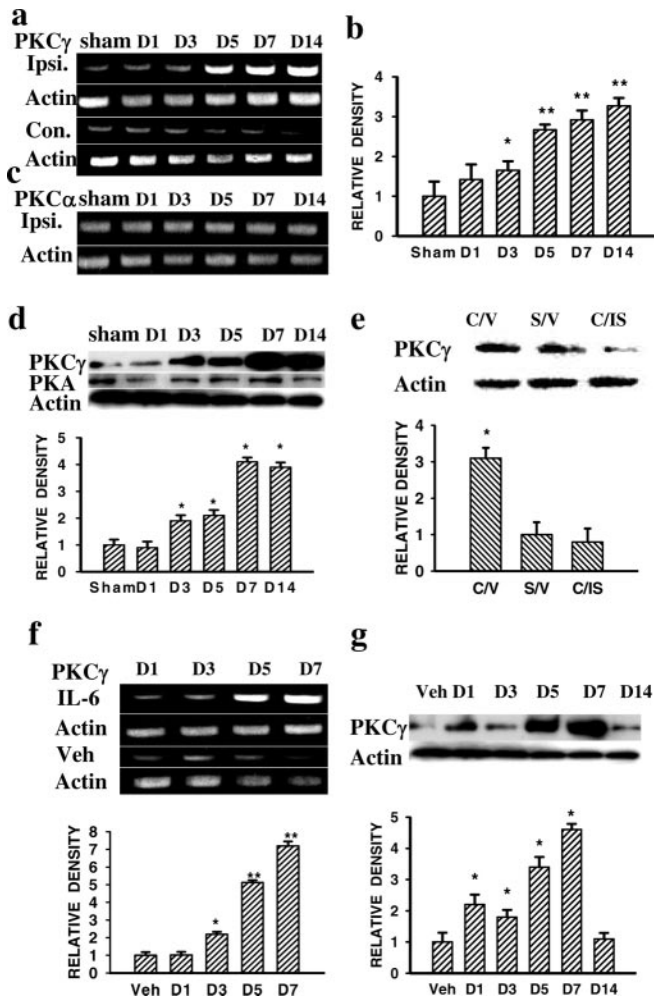
Figure 7. *a–d*, RT-PCR (*a*) and Western blot (*d*) (80 kDa) and statistical analysis (*b*, *d*) showing the time course of changes in PKCγ within the spinal cord dorsal horn of sham and CCI rats. There was no significant change in PKCα (*c*) or PKA (*d*) within the ipsilateral spinal cord dorsal horn after CCI. *$p < 0.05$ and **$p < 0.01$ compared with sham rats (ipsilateral side only). *e*, IL-6 antiserum (5 μg, i.t.) given once daily for the first 6 postoperative days attenuated the expression of PKCγ within the ipsilateral spinal cord dorsal horn of CCI rats. *$p < 0.05$ compared with CCI rats treated with IL-6 antiserum. See Figure 5 for additional labels. *f, g*, RT-PCR (*f*) and Western blot (*g*) and statistical analysis showing the time course of PKCγ expression within the spinal cord dorsal horn of naive rats induced by IL-6 (33 μg) or vehicle (veh) given intrathecally once daily for 6 consecutive days. *$p < 0.05$ compared with vehicle. D1–D7, Day 1 to day 7 of the IL-6 treatment. Note that the GR expression returned to baseline in *g* at 7 d after the discontinuation of IL-6 treatment (D14).
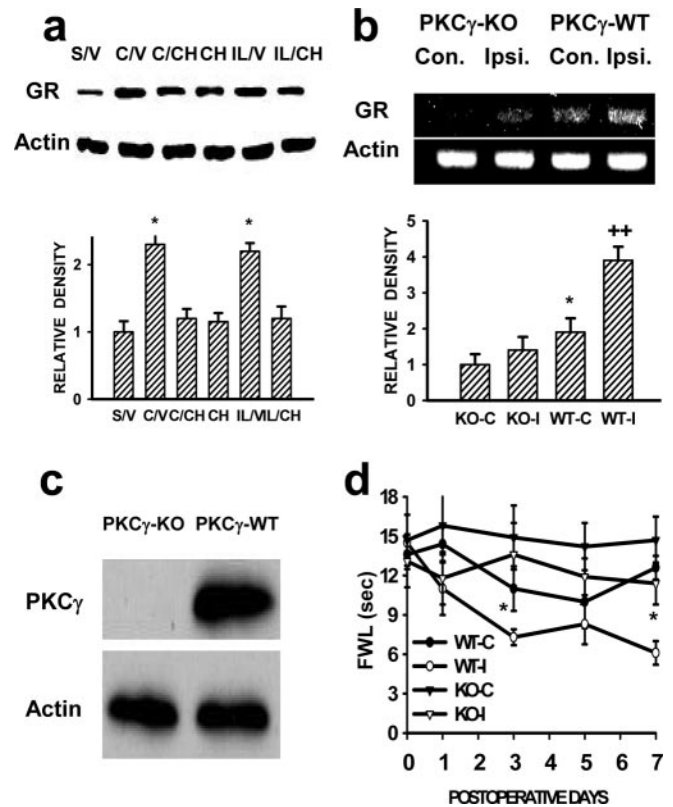

Figure 8. *a*, The PKC inhibitor cheryrithrin (10 μg, i.t.) given twice daily for the first 6 postoperative days attenuated the GR expression within the ipsilateral spinal cord dorsal horn of CCI rats when examined on day 7. The same cheryrithrine treatment also attenuated the GR expression induced by the IL-6 treatment in naive rats. S/V, Sham rats with vehicle; C/V, CCI rats with vehicle; C/CH, CCI rats with cheryrithrine; CH, cheryrithrine alone; IL/V, naive rats with IL-6 and vehicle; IL/CH, naive rats with IL-6 and cheryrithrine. *$p < 0.05$ compared with the corresponding vehicle or sham group. *b*, The GR expression (RT-PCR) after CCI was nearly blocked in PKCγ-KO mice compared with PKCγ-WT when examined on postoperative day 7. *$p < 0.05$ compared with the contralateral spinal cord dorsal horn of PKCγ KO mice (KO-C). ++$p < 0.01$ compared with PKCγ KO mice and the contralateral spinal cord dorsal horn (WT-C) of the WT mice. *c*, Lack of baseline PKCγ expression (Western blot, 80 kDa) within the spinal cord dorsal horn in uninjured PKCγ-KO mice compared with uninjured PKCγ-WT mice. *d*, The development of thermal hyperalgesia was reduced in PKCγ-KO mice compared with PKCγ-WT mice. *$p < 0.05$ compared with PKCγ-WT mice. FWL, Foot-withdrawal latency.

ments confirmed at the tissue harvest, and (2) no GR changes were detected in the ipsilateral DRG and contralateral thalamus after CCI.

The cellular mechanisms of GR actions on the development of neuropathic pain behaviors in CCI rats remain to be elucidated. GR is a cytosolic receptor and activation of GR requires the formation of a GR-homodimer after dissociation from its cytosolic complex consisting of heat shock proteins (Drouin et al., 1992; Neeck et al., 2002). A GR-homodimer binds to nuclear-specific DNA responsive elements and regulates gene transcription and translation for a number of cellular elements (Drouin et al., 1992; Karst et al. 2002). In addition, GR action could take place through nongenomic mechanisms (Barrett and Vedeckis, 1996; Refojo et al., 2001), including (1) the GR-mediated inhibition of bradykinin-induced Ca^{2+} influx in PC12 cells (Qiu et al., 2003), (2) the effect of cortisol through GRs on prolactin release (Borski

et al., 2002), (3) regulation by GRs of glutamate binding in the injured spinal cord (Gonzalez et al., 1995), and (4) a corticosterone-induced acute elevation of intracellular Ca^{2+} concentration in hippocampal neurons (Takahashi et al., 2002). Of interest is that a number of previous studies have suggested that GRs may have modulatory effects on the expression and function of NMDAR. For example, GR activation has been shown to (1) potentiate NMDA-induced responses in dopamine-sensitive neurons within the ventral tegmental area (Cho and Little, 1999) and modulate Ca^{2+} channel conductance (Nair et al., 1998), (2) regulate NMDAR-mediated long-term depression (Coussens et al., 1997) and elevate intracellular Ca^{2+} concentration in hippocampal neurons (Takahashi et al., 2002), and (3) mediate NMDA-induced neuronal degeneration in the rat magnocellular nucleus basalis (Abraham et al., 2000). Given that NMDAR plays a critical role in the mechanisms of neuropathic pain (Mao et al., 1992a, 1995; Yamamoto and Yaksh, 1992; Woolf and Mannion, 1999), it would be of considerable interest in future studies to examine the relationship between GR actions and NMDAR expression and function in relation to the development

as well as maintenance of neuropathic pain behaviors after peripheral nerve injury.

The present findings may have important clinical implications. First, high doses of systemic corticosteroids are often used to reduce signs of regional tissue inflammation such as edema and neurogenic extravasation during the immediate phase of nerve injury (Kingery et al., 1999, 2001). Although the anti-inflammatory effects of corticosteroids could be beneficial under such circumstances, our results indicate that an unexpected adverse effect of GR activation within the CNS could contribute to the neural mechanisms of neuropathic pain behaviors. Second, neuronal degeneration within the spinal cord dorsal horn occurs after peripheral nerve injury (Sugimoto et al., 1990; Mao et al., 1997; Moore et al., 2002), and recent studies have indicated detrimental effects of GR agonists on NMDA-induced neuronal degeneration (Abraham et al., 2000) and in neuronal apoptosis during autoimmune-related inflammation (Diem et al., 2003). Thus, actions of central GRs could potentially worsen neuronal degeneration induced by peripheral nerve injury. Third, endogenous corticosteroids are elevated in chronic pain patients (Moore et al., 1983), and both stress and pain-related responses can activate the hypothalamic–pituitary–adrenocortical axis (Lephart et al., 2003). In this regard, psychological disturbances and chronic stress could influence central nociceptive processing via neuronal GRs, thereby contributing to a long-known clinical connection between psychological and stress responses and pain experience (Dubrovsky, 2000). Our results also suggest potential new clinical treatment for neuropathic pain. For instance, a GR antagonist such as RU38486 could be used to protect the central effects of GR activation. A GR antagonist could also be used in combination with other compounds (e.g., an anti-inflammatory agent, a PKC inhibitor) to reduce chronic neuropathic pain.

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