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Mirror-image pain is mediated by nerve growth factor produced from tumor necrosis factor alpha-activated satellite glia after peripheral nerve injury

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ABSTRACT

Mirror-image pain is characterized by mechanical hypersensitivity on the uninjured mirror-image side. Recent reports favor central mechanisms, but whether peripheral mechanisms are involved remains unclear. We used unilateral spinal nerve ligation (SNL) to induce mirror-image pain in rats. On the mirror-image (contralateral) side, we found that satellite glia in the dorsal root ganglion (DRG) were activated, whereas macrophages/Schwann cells in the DRG and astrocytes/oligodendrocytes/microglia in the dorsal spinal cord were not. Subsequently, an increase in nerve growth factor (NGF) was detected in the contralateral DRG, and NGF immunoreactivity was concentrated in activated satellite glia. These phenomena were abolished if fluorocitrate (a glial inhibitor) was intrathecally injected before SNL. Electrophysiological recordings in cultured small DRG neurons showed that exogenous NGF enhanced nociceptor excitability. Intrathecal injection of NGF into naive rats induced long-lasting mechanical hypersensitivity, similar to SNL-evoked mirror-image pain. Anti-NGF effectively relieved SNL-evoked mirror-image pain. In the contralateral DRG, the SNL-evoked tumor necrosis factor alpha (TNF-α) increase, which started later than in the ipsilateral DRG and cerebrospinal fluid, occurred earlier than satellite glial activation and the NGF increase. Intrathecal injection of TNF-α into naive rats not only activated satellite glia to produce extra NGF in the DRG but also evoked mechanical hypersensitivity, which could be attenuated by anti-NGF injection. These results suggest that after SNL, satellite glia in the contralateral DRG are activated by TNF-α that diffuses from the injured side via cerebrospinal fluid, which then activates satellite glia to produce extra NGF to enhance nociceptor excitability, which induces mirror-image pain.

1. Introduction

For over a century, doctors have reported cases of a mysterious phenomenon: when pain appears in the arm or leg ipsilateral to trauma or inflammation, the patient also senses pain in the contralateral limb, known as mirror-image pain (abbreviated as mirror pain) [2,18,20,25,26,32]. Mirror pain has been also observed in various animal pain models with unilateral nerve injury or inflammation, and mechanical hypersensitivity is the major characteristic [1,9,14,34,36,46]. How is mirror pain induced? Recent studies favor mechanisms in the central nervous system, mainly the activation of astrocytes and microglia in the contralateral dorsal spinal cord [9,14,36,46]. Nevertheless, unilateral nerve injury or inflammation also activates satellite glia and macrophages in the contralateral dorsal root ganglion (DRG) of animals with mirror pain [14,59,62]. Because the DRG is a tissue in the peripheral nervous system (PNS), these studies suggest that satellite glia and macrophages in the PNS may participate in the induction of mirror pain.

Neurotrophic factors produced from areas with nerve injury—including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3), and glial-derived neurotrophic factor (GDNF)—also act as pain mediators [33]. In the DRG of an injured nerve, NGF and NT3 mRNAs are increased

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in satellite glia [63], whereas BDNF protein is increased in neurons [40]. Direct delivery of exogenous NGF or BDNF, but not NT3, to the intact DRG can trigger a persistent mechanical hypersensitivity [64]. Furthermore, GDNF directly sensitizes nociceptors in vitro [31], which may induce pain in vivo. Because the production of neurotrophic factors on the contralateral side is qualitatively similar but quantitatively smaller in scale compared with the injured side [25], whether or which neurotrophic factor is required for the induction of mirror pain needs to be elucidated.

Inhibition of tumor necrosis factor α (TNF-α, an inflammatory cytokine) by intrathecal application of an antagonist can effectively relieve inflammation-induced mirror pain [34,54]. Increased TNF-α levels in the bilateral DRG are accompanied by nerve injury-induced mirror pain [14]. Moreover, crosstalk between TNF-α and NGF exists in immune cells and is implicated in the onset of airway diseases [42]. TNF-α can induce NGF expression in differentiated adipocytes [55] and in astrocyte culture [27]. These studies imply crosstalk between TNF-α and NGF in the induction of mirror pain.

In this report, using an animal model of mirror pain, we found that shortly after unilateral peripheral nerve injury, satellite glia in the contralateral DRG are rapidly activated by an increased TNF-α level and then produce excess NGF to enhance the excitability of small-sized DRG neurons, which results in mirror pain. Accordingly, our data support that mirror pain is induced by a mechanism in the PNS.

2. Materials and methods

2.1. Animals

Male adult Sprague-Dawley rats (200–250 g) were provided by the Animal Center of National Yang-Ming University. All the experimental procedures were approved by the Animal Care Committee of National Yang-Ming University. Rats were housed individually in plastic cages with soft bedding at room temperature and maintained on a 12 hour light/dark cycle with free access to food and water.

2.2. L5/L6 spinal nerve ligation

The spinal nerve ligation (SNL) method was similar to that described previously [24]. The rat (200–250 g on the day of ligation) was anesthetized with inhalational isoflurane (induced with 3% isoflurane in O_2 at 2 L/min and maintained with 0.5% isoflurane in O_2) and placed under a microsurgical apparatus in a prone position. To ligate the spinal nerves on the right side, a midline incision was made along the back, and the right paraspinal muscles were separated from the spinous processes between the L4 and S2 levels. After the L6 transverse process had been removed the L4 and L5 spinal nerves were identified. We tightly ligated the L5 spinal nerve with a 6–0 silk thread at a site 1–2 cm distal to the L5 DRG, and kept holding tight for 10 seconds before making another knot upon the first knot. The L6 spinal nerve on the right side, located caudally and medially to the sacroiliac junction, was ligated similarly. Animals were allowed to recover after closure of the incision. Sham-operated controls were rats receiving the same operation but without nerve ligation.

2.3. Behavioral tests

Thermal and mechanical sensitivity in both hind paws of rats was measured at 2–4 PM on each testing day by an examiner blinded to the treatment groups. After the rat had been habituated to the testing environment daily for at least 2 days, the baseline was measured 1 day before surgery or NGF injection. Six rats were used in each group. Thermal sensitivity was measured by an Analgesia Meter apparatus (IITC/Life Science Instruments) as described previously [13]. The rat was placed on a temperature-controlled glass floor 3-mm thick and habituated for ~30 minutes before testing. A movable light box was located beneath the glass floor, with radiant heat focusing on the planter surface of the hind paw. The light intensity was adjusted to obtain a baseline value of withdrawal within 10–12 seconds. A cutoff time was set at 20 seconds to avoid tissue damage. Three withdrawal latencies were collected with at least 5 minute intervals and the mean represented the thermal latency.

Mechanical sensitivity was assessed by von Frey filaments (Stoelting, Wood Dale, IL). The rat was placed in a transparent plastic dome with a metal-mesh floor allowing access to the plantar surface of the hind paw. There was a habituation time of ~30 minutes before testing. A filament was pressed perpendicularly to the plantar surface of the hind paw for 6 seconds, with sufficient force to cause a slight buckling. A positive response was noted when the hind paw was sharply withdrawn. Flinching immediately upon removal of the filament was also considered a positive response. The force (in grams) producing a 50% likelihood of withdrawal was determined by the "up-down" method as reported previously [4]. Each trial was repeated at 2-minute intervals, and the mean value across 3 trials represented the mechanical threshold (in grams).

2.4. Intrathecal drug administration

All drugs were administrated intrathecally by lumbar puncture, which was modified from Papir-Kricheli et al. [38]. After light anesthesia with inhalation of isoflurane, the rat was placed in a prone position and the midpoint between the tips of the iliac crest was located. A Hamilton syringe with 30-gauge needle was inserted into the subarachnoid space of the spinal cord between the L5 and L6 spinous processes. The preparation of 2 mM fluorocitrate solution has been described previously [6]. Briefly, 8 mg DL-fluorocitric acid barium salt (Sigma, Saint Louis, MO; containing 2 molecules of fluorocitrate) was dissolved in 1 mL of 0.1 M HCl. The barium ion was precipitated with the addition of 2–3 drops of 0.1 M Na_2SO_4. The solution was buffered with 2 mL of 0.1 M NaH_2PO_4 and centrifuged at 800 g for 10 minutes. The supernatant containing the fluorocitrate was diluted with 0.9% NaCl to 2 mM and sterilized by passing through a 0.22 μm syringe filter (Millipore, Billerica, MA). Immediately before ligation, 10 μl of 2 mM fluorocitrate solution was injected. The other solutions for intrathecal injection included 10 μl of phosphate-buffered saline (PBS) containing mouse 7S-NGF ($\alpha_2\beta_2\gamma_2$, Alomone, Jerusalem, Israel; 0, 1, 3, or 10 μg), rabbit anti-mouse NGF-β antibody (10 μg; Millipore), biotinylated nonspecific rabbit anti-mouse IgG (10 μg; Sigma), and either recombinant rat TNF-α (1 or 3 μg; R&D Systems, Minneapolis, MN) or 10 μl of water containing SPD304 (50 μM; Sigma). Each injection was followed by a 10 μl PBS flush. Six rats were used in each group.

2.5. Transcardiac perfusion

After deep anesthesia by intraperitoneal injection of sodium pentobarbital (100 mg/kg), the rat was perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 20 minutes. The bilateral L5 DRG and the spinal cord were isolated and postfixed in the same fixative at room temperature: 30 minutes for DRG and 60 minutes for spinal cord. Following dehydration for cryoprotection in 30% (w/v) sucrose in 0.1 M phosphate buffer (0.08 M K_2HPO_4, 0.02 M NaH_2PO_4, pH 7.4), all specimens were kept at −72 °C before sectioning for immunohistochemistry.

2.6. Single-antigen immunohistochemistry

The L5 DRG in random orientation was cut with a cryostat into 20-μm sections and mounted directly onto gelatin-coated slides. Transverse sections (20 μm) of the L5 spinal cord, in which the contralateral ventral horn was marked by a needle puncture, were processed in a floating manner. After washes in low-salt Tris-buffered saline (LTBS, 25 mM Tris, 0.85% NaCl, pH 7.5) and then LTBS containing 0.3% Triton X-100, sections were treated with 0.3% hydrogen peroxide in LTBS to exhaust endogenous hydrogen peroxidase activity until there were no bubbles. Nonspecific binding was blocked by 3% normal serum (from an animal species the same as the secondary antibody) plus 2% bovine serum albumin in LTBS containing 0.1% Triton X-100 (LTBST) for 1.5 hours. Sections were incubated overnight at room temperature with primary antibodies in LTBST containing 3% normal serum. Primary antibodies were mouse anti-CNPase (1:1000; Sigma), rabbit anti-GFAP (1:3000; Dako, Glostrup, Denmark), rabbit anti-Iba1 (1:800; Wako, Osaka, Japan), rabbit anti-NGF (1:200; Alomone), and goat anti-TNFR1 (1:50; R&D Systems). Sections were then washed with high-salt Tris-buffered saline (50 mM Tris, 1.7% NaCl, pH 7.5) containing 0.1% Triton X-100 and then incubated for 1.5 hours with biotinylated donkey anti-goat IgG (1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-rabbit IgG (1:1000; Pierce, Rockford, IL), or horse anti-mouse IgG (1:500; Vector, Burlingame, CA). Following incubation with avidin-biotin horseradish peroxidase complex (1:160; Pierce) for 1.5 hours, antigen was visualized by combining equal volumes of ammonium nickel sulfate (30 mg/mL in 0.1 M sodium acetate, pH 6.0) and diaminobenzidine (4 mg/mL in LTBS) in the presence of 0.01% hydrogen peroxide. Floating sections of spinal cord were spread flat on slides and air dried. Sections on slides were dehydrated through an ethanol gradient/xylene and coverslipped with a mounting medium (Permount; Merck, Darmstadt, Germany). Images were acquired using the BX51 light microscope (Olympus, Tokyo, Japan) and processed with Photoshop CS2 software (Adobe Systems).

2.7. Western blotting

The rat was decapitated after brief isoflurane anesthesia. DRG and spinal cord were frozen on dry ice immediately after isolation. The L5 and L6 DRG on the same side were processed together. The dorsal spinal cord in the L5–L6 region was separated into the right and left parts. Tissues were cut into small pieces and homogenized in ice-cold lysis buffer (20 mM Tris [pH 8.0], 137 mM NaCl, 1% NP-40, and 10% glycerol) containing 0.5 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 20 μM calpain inhibitors I and II (Sigma), and 1% protease inhibitor cocktail [1.04 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 800 nM aproptinin, 40 μM bestatin hydrochloride, 14 μM N-(trans-epoxysuccinyl)-L-leucine-guanidinobutylamide, 20 μM leupeptin hemisulfate salt, and 15 μM pepstatin A] (Sigma). The supernatant was collected after centrifugation at 14,000 rpm for 10 minutes at 4 °C to remove debris, the protein concentration was determined by the BCA protein assay (Thermo, Rockford, IL), and aliquots of the supernatant were stored at −72 °C before use. Sample solution contained approximately 20 μg of proteins in a loading buffer (50 mM Tris [pH 6.8], 2% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, and 10% glycerol). After denaturing by boiling for 5 minutes in the presence of 5% 2-mercaptoethanol, proteins were separated by 12% (for GFAP, Iba1, and CNPase) or 15% (for NGF, BDNF, and GDNF) SDS/polyacrylamide gel electrophoresis, and electrophoretically transferred to a nitrocellulose membrane using a semi-dry method (Nova Blot, Pharmacia Biotech). After a brief rinse in PBS containing 0.05% Tween 20 (PBSW), the membrane was blocked for nonspecific binding with 5% nonfat milk in PBSW for 1 hour, washed in PBSW 3 times for 10 minutes, and incubated with primary antibody in PBSW containing 1% bovine serum albumin at 4 °C overnight.

Primary antibodies included rabbit anti-BDNF (1:500; GeneTex, Irvine, CA), mouse anti-CNPase (1:1000; Sigma), rabbit anti-GDNF (1:1000; Santa Cruz Biotech, Dallas, TX), anti-GFAP (1:10000; Dako), rabbit anti-Iba1 (1:2000; Wako), rabbit anti-NGF (1:250; Alomone), and goat anti-TNF-α (1:500; R&D Systems). Following washing, donkey anti-goat, goat anti-mouse, or goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10000; Jackson ImmunoResearch Laboratories) was applied to the membrane for 1 hour at room temperature. In the presence of a chemiluminescent reagent (Luminata Forte, Millipore), immunoreactive bands were visualized by exposing a membrane in a luminescent image analyzer and images were acquired (Fujifilm LAS-4000, Tokyo, Japan). The membrane was then washed using a stripping buffer (0.4 M glycine [pH 2.0], 0.2% SDS, and 2% Tween 20) and re-immunoblotted with a rabbit antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:10000; GeneTex) as a control of total proteins for normalization. The immunoreactivity of each band of the corresponding molecular weight position in each image was circled and measured by Image J 1.40c software (U.S. National Institutes of Health): BDNF (3 bands at 14 [mature form], 45 and 70 kDa [immature forms] were detected and only the band at 14 kDa was measured), CNPase (only a single band at 46 kDa was detected), GAPDH (only a single band at 36 kDa was detected), GDNF (only a single band at 40 kDa was detected), GFAP (2 bands at 49 kDa [unphosphorylated form] and 50 kDa [phosphorylated form] were detected and both bands were measured), Iba1 (only a single band at 17 kDa was detected), NGF (only a single band at 13 kDa [mature form] was detected), and TNF-α (only a single band at 17 kDa was detected).

2.8. Double-label fluorescent immunohistochemistry

DRG sections were processed in a similar manner to that described in Section 2.6, except that the pretreatment with hydrogen peroxide was omitted and sections were incubated simultaneously with 2 of the following primary antibodies: mouse anti-GFAP (1:5000; Sigma), guinea pig anti-GLAST (1:1000; Millipore), rabbit anti-NGF (1:100; Alomone), and goat anti-TNFR1 (1:25; R&D Systems). Secondary antibodies included Alexa Fluor 488-conjugated donkey anti-goat (1:200; Invitrogen, Eugene, OR), Rhodamine Red-X-conjugated donkey anti-guinea pig (1:200; Jackson ImmunoResearch Laboratories), Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:500; Invitrogen), and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200; Invitrogen). After LTBS wash, sections were mounted with an antifading medium (Fluoromount-G; Southern Biotech, Birmingham, AL) under coverslips. Images were collected using the FV300 confocal laser scanning microscope (Olympus) and processed with Photoshop CS2 software.

2.9. Electrophysiology

The rat was decapitated after brief isoflurane anesthesia, and the bilateral L5/6 DRG were isolated aseptically. Following dissociation using 1 mg/mL type IA collagenase (Sigma) for 1.5 hours and then 0.25% trypsin (Invitrogen) for 30 minutes, cells were plated onto 12 mm-diameter coverglasses coated with poly-L-lysine (Sigma) and laminin (Invitrogen). DRG cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 100 U penicillin/100 μg streptomycin (Sigma) per ml, in a humid chamber with 5% CO_2 at 37 °C. NGF (100 ng/mL) was added to some cells 1 hour after plating, and all cells were cultured for another 16–20 hours before testing. A coverglass with cells was transferred to a chamber and

cells were constantly perfused with a buffer containing 145 mM NaCl, 3 mM KCl, 10 mM glucose, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 10 mM HEPES (pH adjusted to 7.3 with KOH). The pipette solution for whole-cell patch-clamp recordings contained 135 mM potassium gluconate, 20 mM KCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 4 mM Na_2ATP and 10 mM HEPES (pH adjusted to 7.3 with KOH). Small DRG neurons (soma diameter $\leqslant$30 μm) were visually selected for recording under bright-field optics (BX51WI, Olympus). Using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), whole-cell patch-clamp recordings were made at 22–24 °C as described previously [29]. Patch pipettes (2–5 MΩ) were pulled from borosilicate glass tubing (outer diameter 1.5 mm and inner diameter 0.86 mm; Harvard apparatus, Holliston, MA) and heat polished. Pipette capacitance was compensated using values slightly lower than those determined previously in the cell-attached configuration. Series resistance (10–40 MΩ) was compensated using the automatic bridge balance. Spikes were evoked in single neurons by 1-second current pulse (100–600 pA) injection in the current-clamp configuration. Spike number was calculated from a 1-second spike train elicited in response to current pulse injection. Signals were low-pass filtered at 4 kHz (four-pole Bessel) and sampled at 10 kHz using the Digidata 1440 interface (Molecular Devices). Data acquisition and pulse generation were performed using pClamp 10.2 software (Molecular Devices).

2.10. Cerebrospinal fluid sampling and TNF-α ELISA

The method for cerebrospinal fluid (CSF) sampling was modified from De la Calle and Paino [7]. The rat was anesthetized with inhalational isoflurane and placed in a prone position on a Styrofoam board (20 cm × 17 cm × 3 cm). The rat's forelimbs were extended towards the front and the hind limbs were left to hang off the board, lying on the table. In that way, the animal's vertebral column was flexed around the L3 to L5 levels, widening these intervertebral spaces. The anterior part of the iliac crest was used as a tactile landmark for the L5 to L6 intervertebral levels. After shaving, a neonatal lumbar puncture needle (25G × 1, Terumo, Tokyo, Japan) was inserted through the L4–5 intervertebral space into the intrathecal space. A short tail flick was observed upon needle insertion. CSF spontaneously flowed out to the needle cup and was collected into a tube. After a brief spin to pellet suspended cells, the supernatant was collected to another tube. The CSF samples were frozen at −72 °C before use. For each SNL rat, CSF was collected at the following 4 time points: immediately before SNL (0 hours), and 6 hours, 12 hours, and 1 day after SNL. At least 20 μL of CSF were collected from one rat at each time point. Then, the TNF-α protein level in the CSF supernatant was measured by a rat TNF-α ELISA kit (Thermo) according to the manufacturer's procedure. Values for the CSF samples and the standard curve were obtained from the optical density at 450 nm by an ELISA reader (Tecon, Männedorf, Switzerland). The mean concentration of TNF-α at 0 hours was set at 1 and concentrations at the other time points were represented as "fold of 0 hours" for each rat. The data were collected from 3 rats.

2.11. Statistics

SPSS 18.0 for Windows (IBM, Chicago, USA) was used for the statistical calculations. Values obtained from the behavioral tests (n = 6) and western blot (n = 3) were presented as means ± SEM. Differences between groups were compared by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference post hoc test (for behavioral tests) or Student's *t* test (for western blot). For electrophysiology, differences between groups were compared by Student's *t* test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for all analyses.

3. Results

3.1. Induction of mirror pain after peripheral nerve injury

The unilateral L4–L6 spinal nerves of the rat merge into the sciatic nerve, the primary nerve of the hind limb. To evoke mirror pain in the left hind limb, we tightly ligated the L5 and L6 spinal nerves on the right side. In the sham-operated rats, bilateral mechanical and thermal sensitivity showed no significant change compared with the baseline (Fig. 1A, B). In the rats with unilateral SNL, bilateral mechanical hypersensitivity appeared at day 1 and was sustained during days 2–7 (Fig. 1A), whereas thermal hypersensitivity was observed only on the injured side during days 1–7 (Fig. 1B). This SNL model consistently evoked mechanical hypersensitivity on the uninjured mirror-image side (ie, mirror pain), similar to previous studies [1,9,14,34,36,46].

Fluorocitrate has been used as a glial metabolic inhibitor in pain-related studies [30,34,48,51]. Intrathecal administration of fluorocitrate immediately before applying an immune activator around 1 sciatic nerve of rats can prevent the development of inflammation-evoked mirror pain [34]. To test whether it also works in this SNL model, we intrathecally injected fluorocitrate into rats 30 minutes before SNL. A single fluorocitrate injection effectively delayed the development of SNL-evoked mirror pain for 2 days, in addition to attenuating mechanical hypersensitivity on the injured side (Fig. 1C, D). It suggests that glial cells in the spinal cord and/or DRG, which are accessible by drugs administrated intrathecally, may be involved in the induction of mirror pain.

3.2. Slow glial activation in the contralateral dorsal spinal cord

Previous reports suggest that glial activation in the dorsal spinal cord is crucial for the induction of mirror pain [9,34,36,46]. To clarify whether glial activation or microglial recruitment in the dorsal spinal cord is required for the induction of mirror pain, we combined immunohistochemistry and western blot to analyze the expression levels of glial fibrillary acidic protein (GFAP, for astrocytes), ionized calcium-binding adaptor molecule 1 (Iba1, for microglia), and cyclic nucleotide phosphodiesterase (CNPase, for oligodendrocytes). In the contralateral L5/L6 dorsal spinal cord of SNL rats, GFAP did not increase at day 1 but showed a 3-fold increase at day 7, whereas Iba1 and CNPase levels had no significant increase at day 1 or day 7 (Fig. 2), indicating that only astrocytes were activated at day 7. Because glial activation in the contralateral dorsal spinal cord was later than the appearance of mirror pain, spinal glia could not be responsible for the induction of mirror pain.

3.3. Rapid and dramatic activation of satellite glia in the contralateral DRG

To elucidate whether glial cells and macrophages in the PNS are involved in the induction of mirror pain, we performed immunohistochemistry in the DRG by labeling GFAP for satellite glia, Iba1 for macrophages, and CNPase for Schwann cells. In the contralateral L5 DRG for 1–7 days after SNL, we found an increase in GFAP at day 1 and day 7 (Fig. 3A), an increase in Iba1 at day 7 (Fig. 3B), but no significant change in CNPase (Fig. 3C). One day after intrathecal fluorocitrate injection and SNL, we found that fluorocitrate inhibited the SNL-induced GFAP increase in the contralateral L5 DRG but had no effect on the Iba1 or CNPase expression level (Fig. 3A–C). Similar expression patterns for these 3 markers were detected in the contralateral L6 DRG (data not shown).

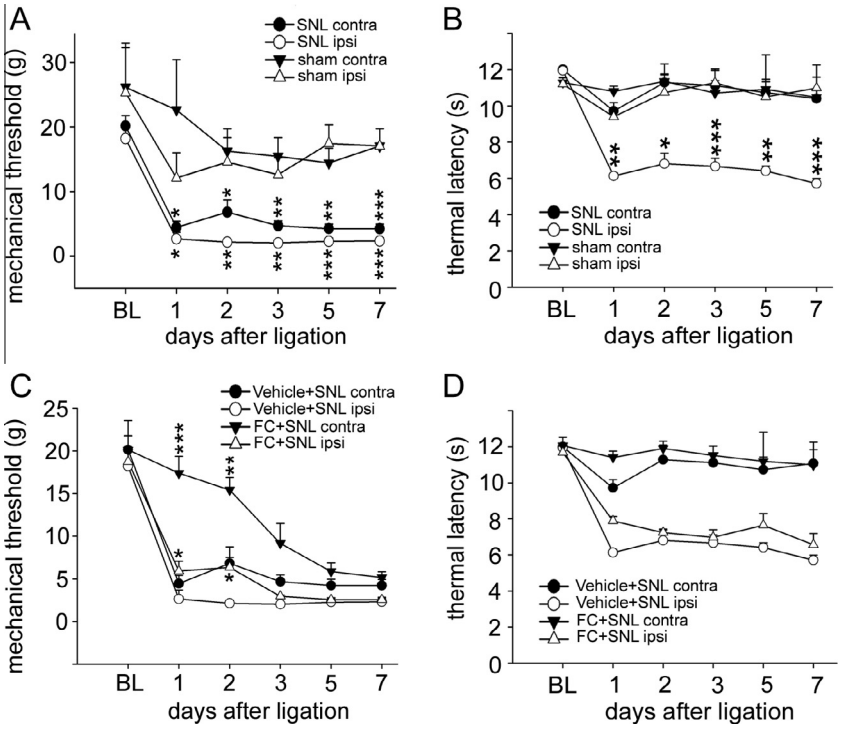

Fig. 1. Glial inhibitor pretreatment delays nerve injury-evoked mirror pain. (A, B) The unilateral L5 and L6 spinal nerves of rats were ligated. (A) Mechanical hypersensitivity was observed on the contralateral and ipsilateral sides at day 1. (B) Thermal hypersensitivity appeared only on the ipsilateral side. (C, D) A single dose of fluorocitrate (FC) or vehicle was intrathecally injected 30 minutes before ligation. (C) Fluorocitrate caused a delayed development of contralateral mechanical hypersensitivity (mirror pain) and attenuated ipsilateral mechanical hypersensitivity for 2 days after spinal nerve ligation (SNL). (D) Fluorocitrate pretreatment did not affect thermal hypersensitivity. BL, baseline (1 day before ligation). n = 6; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for the comparison with the corresponding side of the sham-operated (A, B) or vehicle-treated (C, D) group at each indicated day by Tukey's post hoc test after one-way analysis of variance.

The protein levels of GFAP, Iba1, and CNPase in the DRG of SNL rats were quantified by western blot. Because there was only a tiny amount of total protein in a single DRG, the L5 and L6 DRG on each side were pooled together. We found that GFAP was increased to 6-fold on both sides at day 1, and by 8-fold on the contralateral side and 16-fold on the ipsilateral side at day 7 (Fig. 3D, E). In addition, Iba1 was increased to 2-fold and 4-fold, respectively, on the contralateral and ipsilateral sides at day 7 (Fig. 3D, F). There was no significant change in CNPase on either side at day 1 or day 7 (Fig. 3D, G). Intrathecal fluorocitrate injection selectively suppressed the SNL-induced GFAP increase, but had no effect on Iba1 and CNPase levels (Fig. 3E–G). These data indicate that within the first week after SNL, satellite glia were rapidly activated, macrophages were slowly activated, and Schwann cells were not activated.

Comparing the changes between the DRG and dorsal spinal cord on the contralateral side (Figs. 2 and 3), glial activation was faster and larger in scale than macrophage/microglia recruitment, and DRG satellite glial activation occurred earlier and more dramatically than spinal astrocyte activation.

3.4. Rapid and dramatic increase in NGF in the contralateral DRG

To investigate which neurotrophic factor is the major proinflammatory mediator for SNL-evoked mirror pain, we examined NGF, BDNF, and GDNF protein levels in L5/L6 DRG, hind paw skin (innervated by the peripheral sensory axon terminals), and dorsal spinal cord. Although NT3 has been reported to be upregulated in satellite glia of the DRG of an injured spinal nerve [63], we did not examine NT3 because exogenous NT3 could not trigger mechanical hypersensitivity [57,64]. As revealed by western blot analysis, there was a 13-fold increase of NGF in the contralateral L5/L6 DRG at day 1 and day 7 after SNL, which could be abolished by fluorocitrate pretreatment (Fig. 4A, D). By contrast, NGF in the contralateral hind paw skin and dorsal spinal cord showed no significant increase at day 1 or day 7 (Fig. 4B, C, E, F). An increase in BDNF on the contralateral side was detected until day 7: 4-fold in the L5/L6 DRG, 2-fold in the hind paw skin, and 1.5-fold in the dorsal spinal cord (Fig. 4A–C, G–I). There was no significant increase in GDNF in these contralateral regions at day 1 or day 7 (data not shown). Thus, the increase in NGF occurred more quickly and on a larger scale in the contralateral DRG than in the dorsal spinal cord and hind paw skin, and NGF was increased earlier and more dramatically than BDNF.

The expression of the high-affinity NGF receptor TrkA in adult DRG is restricted to nociceptors [8]. After NGF binds to TrkA on nociceptors, mitogen-activated protein kinases are activated [17] and then phosphorylate voltage-gated sodium channels to make nociceptors hyperexcitable without de novo protein synthesis [19,50]. NGF, as well as other neurotrophins, did not significantly alter the number of TrkA(+) DRG neurons at day 3 after intrathecal injection to naive rats [35]. Using western blot analysis, we found that the TrkA level in the contralateral L5/L6 DRG was not changed at day 1 after SNL, but increased to approximately 4-fold at day 7 (Supplementary Fig. 1). It suggests that the basal expression level of TrkA on nociceptors is sufficient to respond to the high NGF concentration in the contralateral DRG and to induce mirror pain at day 1 after SNL.

3.5. High NGF level in activated satellite glia of the contralateral DRG

NGF mRNA has been shown to be highly expressed in satellite glia of the DRG of an injured spinal nerve [63]. The dramatic increase in NGF (Fig. 4D) coincided with satellite glial activation

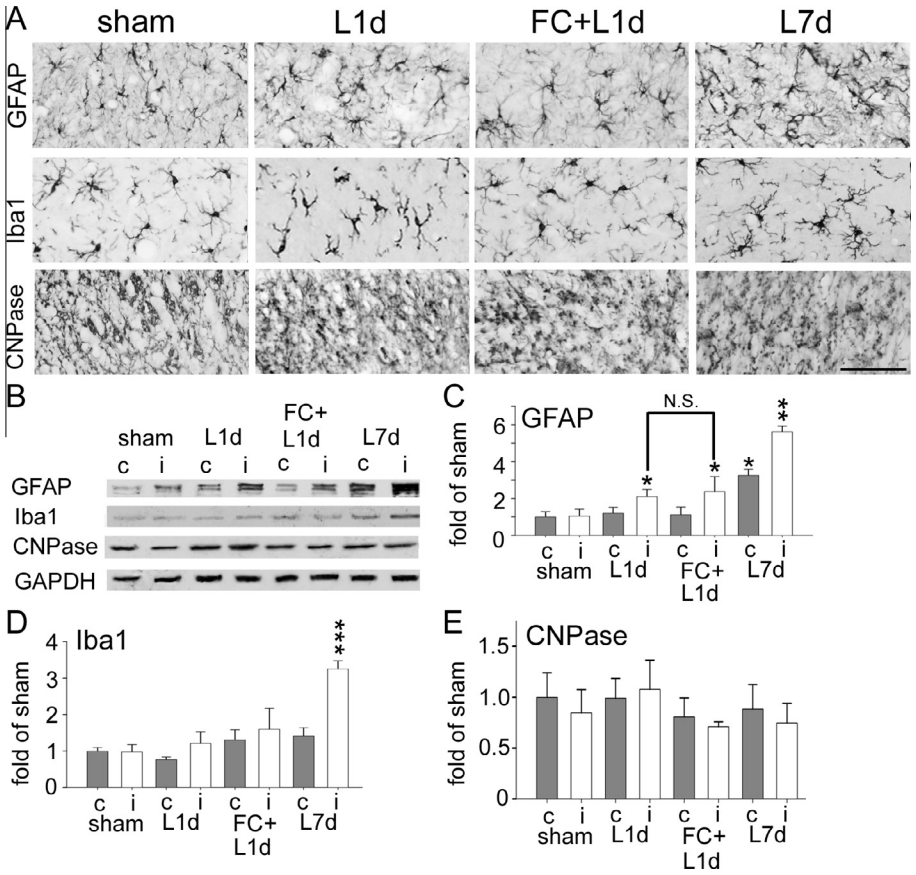

Fig. 2. Slow and slight activation of astrocytes in the contralateral dorsal spinal cord. Rats were sacrificed 1 day (L1d) or 7 days (L7d) after spinal nerve ligation. "FC+L1d" indciates intrathecal injection of fluorocitrate 30 minutes before ligation. (A) Immunostaining of glial fibrillary acidic protein (GFAP, for astrocytes), ionized calcium-binding adaptor molecule 1 (Iba1, for microglia), and cyclic nucleotide phosphodiesterase (CNPase, for oligodendrocytes) in the L5 dorsal spinal cord are shown. Scale bar (shown in B): 30 μm. (B–E) Protein levels of GFAP, Iba1, and CNPase in the contralateral (c) and ipsilateral (i) L5/L6 dorsal spinal cord segment were analyzed by western blot. The representative signals are shown in B, and the quantitative data are shown in C–E. n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test) for the comparison with the contralateral L5 dorsal spinal cord segment of the sham-operated group. There was no significant difference (N.S. in C) in GFAP expression between the L1d and FC+L1d groups, indicating that fluorocitrate injection did not affect astrocytes in the ipsilateral dorsal spinal cord.

in the contralateral DRG (Fig. 3E), suggesting a correlation between NGF and satellite glia. To identify which type of DRG cells produce NGF, we performed immunohistochemistry in the contralateral L5 DRG of rats with SNL for 1 day. Strong NGF immunoreactivity (NGF-IR) was detected in many punctate structures surrounding the somata of DRG neurons, whereas weak NGF-IR was observed in the somata of small- and medium-diameter DRG neurons (Fig. 5A–C). The appearance of NGF-IR could be prevented by fluorocitrate pretreatment (Fig. 5D). Satellite glia are located around the somata of DRG neurons, and double fluorescence immunostaining showed that NGF was expressed abundantly in GFAP(+) satellite glia of the contralateral DRG (Fig. 5E–H). By contrast, there was little NGF-IR in Iba1(+) macrophages and CNPase(+) Schwann cells (data not shown). Thus, a high level of NGF was produced by activated satellite glia in the contralateral DRG shortly after SNL.

3.6. NGF sensitizes nociceptors

Small-diameter neurons in the DRG are known as pain-sensing neurons or nociceptors [11]. Because a high NGF level was detected in the contralateral DRG after SNL (Fig. 4D), we asked whether nociceptor excitability was enhanced after NGF incubation. In small DRG neurons that had been cultured with NGF, although the resting membrane potential was not affected [22,61], the evoked spike number was increased after a 6-minute exposure to NGF in the bath [61]. To mimic the high NGF level detected in the contralateral DRG at day 1 after SNL, we dissociated the DRG of naive rats and cultured them in the presence or absence of NGF for 16–20 hours. Then, without the bath application of NGF, the membrane excitability of small (⩽30 μm diameter) DRG neurons was measured by whole-cell patch-clamp recording. When given a depolarizing current pulse (600 pA, 1 second) injection, multiple spikes were observed in 23% of small DRG neurons that had been cultured without NGF. The percentage of small DRG neurons that generated multiple spikes in response to the same stimulus significantly increased to 48% after NGF treatment (Fig. 6A–D). Furthermore, the input–output relations showed that small DRG neurons after NGF treatment had a significantly greater response to the weak stimulus (200 pA) than those without NGF treatment (3.36 ± 0.68 Hz, n = 89 vs 1.10 ± 0.29 Hz, n = 40) (Fig. 6E). Notably, NGF treatment did not change their responses to stronger stimuli (300–600 pA), indicating that NGF treatment decreased the offset (current threshold), but not the gain control (slope), of the input–output relations. Taken together, NGF treatment for nearly 1 day significantly enhanced the intrinsic excitability of small DRG neurons. These in vitro studies suggest that nociceptors in the contralateral DRG of living animals, which become sensitized after nearly 1 day's exposure to a high level of endogenous NGF, can evoke a greater pain response from a weak stimulus.

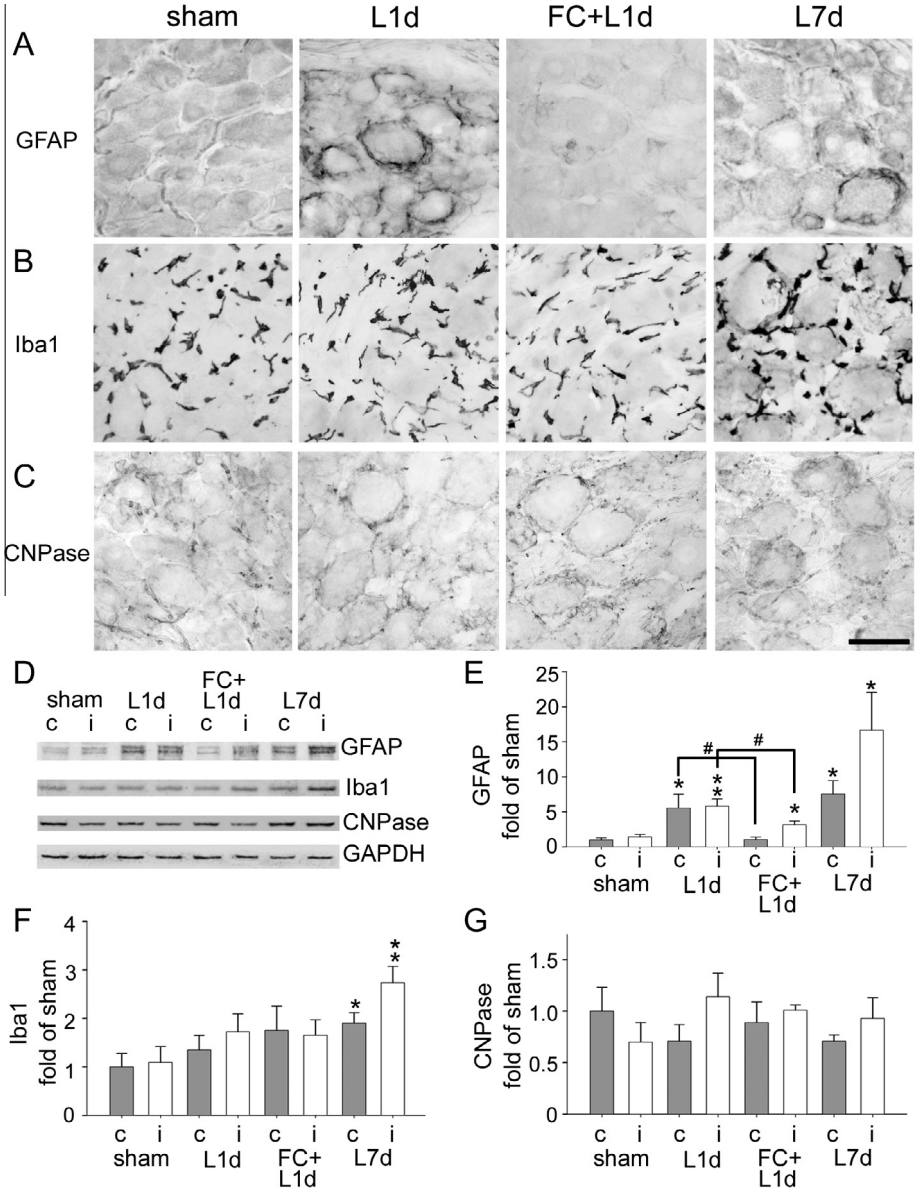

Fig. 3. Rapid and dramatic activation of satellite glia in the contralateral dorsal root ganglion (DRG). Rats were sacrificed 1 day (L1d) or 7 days (L7d) after spinal nerve ligation. "FC+L1d" indicates intrathecal injection of fluorocitrate 30 minutes before ligation. (A–C) Sections of the contralateral L5 DRG were immunostained for glial fibrillary acidic protein (GFAP, for satellite glia; A), ionized calcium-binding adaptor molecule 1 (Iba1, for macrophages; B), or cyclic nucleotide phosphodiesterase (CNPase, for Schwann cells; C). (D–G) Protein levels of GFAP, Iba1, and CNPase in the contralateral (c) and ipsilateral (i) L5/L6 DRG were analyzed by western blot. Representative signals are shown in D, and the quantitative data are shown in E–G. n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t* test) for the comparison with the contralateral L5/L6 DRG of the sham-operated group. $^{\#}P < 0.05$ for the comparison of the indicated pairs. Scale bar (shown in C): 60 μm. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

3.7. Exogenous NGF induces long-lasting mechanical hypersensitivity in naive rats

Since NGF can increase nociceptor excitability in vitro (Fig. 6), we asked whether a higher NGF concentration in the lower lumbar DRG is sufficient to induce pain in the hind paw of living animals. Before injecting NGF (a 130 kDa protein), we tested whether isolectin B4 (IB4, a glycoprotein of 114 kDa, similar to the molecular weight of NGF) can permeate the L5 DRG after intrathecal injection. In the L5 DRG of rats intrathecally injected with IB4 for 6 hours or 1 day, we detected strong IB4-IR in glial cells surrounding neurons and weak IB4-IR in small DRG neurons compared with the rats injected with vehicle (Supplementary Fig. 2A–C). By contrast, there was no IB4 signal inside the L5 spinal cord, except for the surface (Supplementary Fig. 2D, E). Thus, proteins administered by intrathecal injection can permeate into the DRG much more easily than into the spinal cord.

To mimic the increase in endogenous NGF in the contralateral L5 DRG 1 day after SNL, we intrathecally injected 1, 3, or 10 μg NGF into naive rats. There was no significant change in mechanical hypersensitivity in rats receiving vehicle or 1 μg NGF (Fig. 7A, B). In rats receiving 3 or 10 μg NGF, bilateral mechanical hypersensitivity first appeared at day 1 and was sustained for at least 1 week (Fig. 7C, D); bilateral thermal hypersensitivity was observed only at 4 hours and 8 hours in rats receiving 10 μg NGF (Supplementary Fig. 3). Moreover, there was no glial activation in the L5 spinal dorsal horn after intrathecal NGF injection into naive rats (Supplementary Fig. 4). These data demonstrate that the NGF increase in the DRG is sufficient to induce long-lasting mechanical hypersensitivity, the major characteristic of mirror pain.

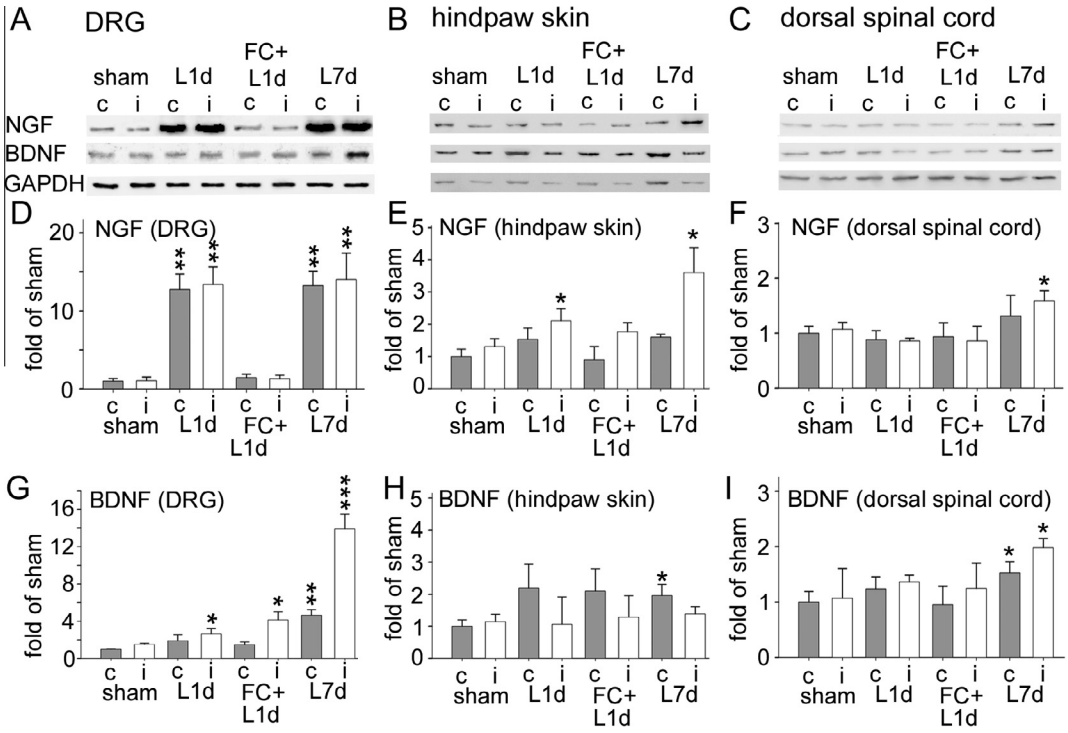

Fig. 4. Rapid and dramatic increase in nerve growth factor (NGF) in the contralateral dorsal root ganglion (DRG). Rats were sacrificed at 1 day (L1d) or 7 days (L7d) after spinal nerve ligation. "FC+L1d" indicates intrathecal injection of fluorocitrate 30 minutes before ligation. Western blots were used to analyze the protein levels of NGF, brain-derived neurotrophic factor (BDNF), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, as an internal control) in the contralateral (c) and ipsilateral (i) sides of the L5/L6 DRG (A, D, G), the hind paw skin (B, E, H), and the L5/L6 dorsal spinal cord segment (C, F, I). The representative signals are shown in A–C, and the quantitative data are shown in D–I. n = 3; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test) for the comparison with the contralateral side of the sham-operated group.

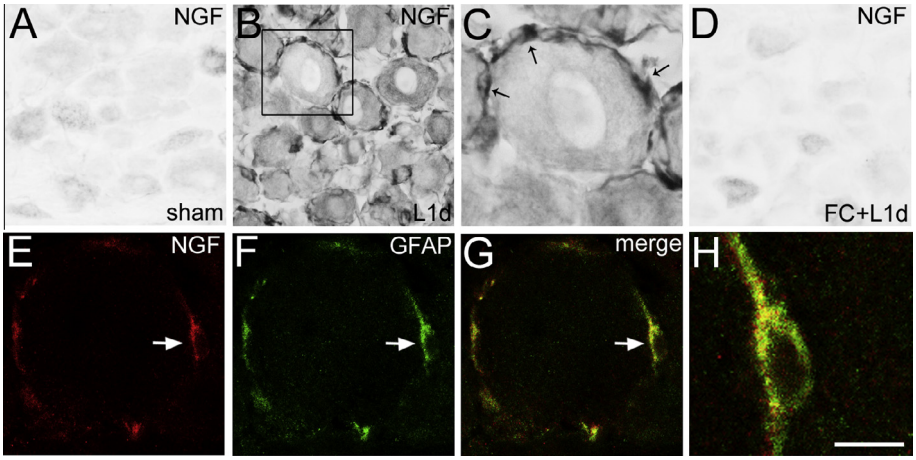

Fig. 5. A high nerve growth factor (NGF) level in activated satellite glia. Rats were sacrificed 1 day after sham operation, spinal nerve ligation (SNL; L1d), or fluorocitrate (FC) injection plus SNL (FC+L1d), and sections of the L5 dorsal root ganglion (DRG) were immunostained for NGF (A–E) and glial fibrillary acidic protein (GFAP; F). (A) Weak NGF immunoreactivity (NGF-IR) in sham-operated rats. (B) Strong NGF-IR in punctate structures surrounding the somata of DRG neurons. (C) A higher magnification of the square in (B), showing NGF-IR in satellite glia (arrows). (D) Induction of NGF in satellite glia was prevented if fluorocitrate was intrathecally injected 30 minutes before SNL. (E–H) Confocal images show that NGF was highly expressed in GFAP(+) satellite glia, and one cell (arrows in E–G) is magnified in H. Scale bar (shown in H): 35 μm (A, B, D); 20 μm (C); 15 μm (E–G); 5 μm (H).

3.8. Anti-NGF can relieve mirror pain

To test whether NGF is the major mediator of mirror pain, NGF antibody (anti-NGF) was used to neutralize excess endogenous NGF produced after SNL. A single dose of 10 μg anti-NGF was injected into the lower lumbar region of the rat at day 4 after SNL, when mirror pain had been induced. Although anti-NGF did not attenuate neuropathic pain (mechanical and thermal hypersensitivity on the injured side) in the subsequent 5 days (days 5–9), it quickly relieved mirror pain (mechanical hypersensitivity on the contralateral side) on the next day (day 5; Fig. 7E). Nonspecific immunoglobulin G (IgG, as a negative control) did not evoke any behavioral change (Fig. 7F). Quick relief by anti-NGF supports NGF as the major mediator of mirror pain.

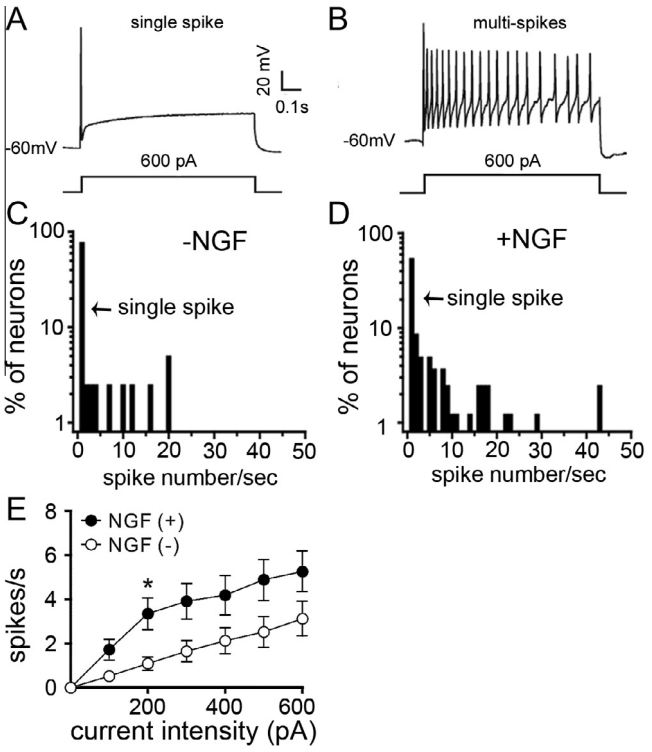

Fig. 6. Nerve growth factor (NGF) enhances the excitability of small dorsal root ganglion (DRG) neurons. Electrophysiological recording was performed in small DRG neurons after culturing with or without NGF for 16–20 hours. In response to the depolarizing current pulse (600 pA; 1 second), small DRG neurons exhibited either a single spike (A) or multiple spikes (B). Overall, multiple spikes were observed in 23% (9 of 40 cells) of small neurons cultured without NGF (−NGF; C) and in 48% (39 of 81 cells) of small neurons cultured with NGF (+NGF; D). (E) Spikes were induced in single neurons by 1-second current pulse (100–600 pA) injection in the current-clamp configuration. Note that the weak stimulus (200 pA) significantly evoked more spikes in NGF-treated neurons (+NGF) compared with nontreated neurons (−NGF). n = 3 experiments per group. $^{*}P < 0.05$ (Student's t test) for the comparison with nontreated neurons injected with the same current intensity.

3.9. Endogenous TNF-α increased in the bilateral DRG and CSF

To investigate whether TNF-α contributes to the induction of mirror pain, we quantified TNF-α levels in the DRG and spinal cord by western blot analysis. At 3 hours after SNL, there was a 3.5-fold increase in TNF-α in the ipsilateral DRG, but no significant change in the contralateral DRG (Fig. 8A). At 6 hours after SNL, TNF-α was increased to 5- and 2.5-fold, respectively, in the ipsilateral and contralateral L5/L6 DRG (Fig. 8A). TNF-α levels in the bilateral DRG were further increased at 12 hours and 1 day, and the inhibition of satellite glial activation by fluorocitrate did not suppress the TNF-α increase (Fig. 8A). However, in the L5/L6 dorsal spinal cord there was a 2.2-fold increase in the ipsilateral side at 1 day whereas the contralateral side showed no significant increase at 6 hours, 12 hours, or 1 day (Fig. 8B).

The concentration of TNF-α in CSF is increased after spared nerve injury [41]. Particularly, mirror pain and TNF-α upregulation in the contralateral DRG have been observed after spinal nerve crush [11]. Based on these reports, to test the possibility that TNF-α can diffuse from the ipsilateral to the contralateral DRG via CSF, we measured TNF-α in CSF. ELISA revealed that TNF-α was increased 2.6-fold at 3 hours and approximately 4-fold at 6 hours, 12 hours, and 1 day after SNL (Fig. 8C). There was no significant increase of TNF-α in CSF after sham operation (data not shown). Because TNF-α was increased first in the ipsilateral DRG and CSF at 3 hours and then in the contralateral DRG at 6 hours, it is likely that the TNF-α increase in the contralateral DRG is due to diffusion from the ipsilateral DRG.

3.10. TNF-α increase is earlier than satellite glial activation and NGF rise in the contralateral DRG

To elucidate whether there is a causal relationship between the TNF-α increase and the NGF rise in the contralateral DRG, in addition to NGF we also quantified GFAP, which reflects the extent of satellite glial activation that results in NGF production.

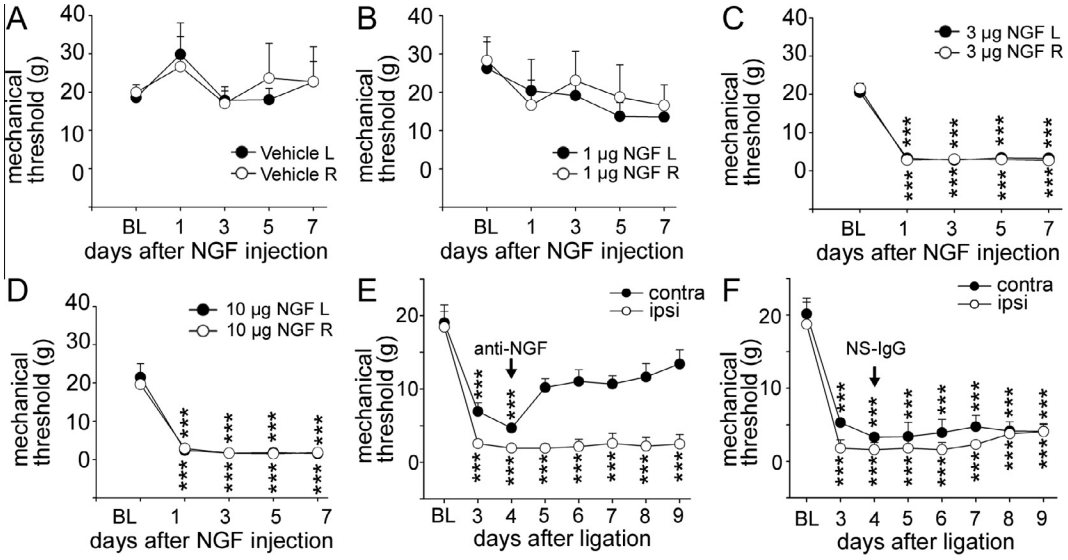

Fig. 7. Nerve growth factor (NGF) induces long-lasting mechanical hypersensitivity. (A–D) Vehicle (A), NGF 1 μg (B), 3 μg (C), or 10 μg (D) was injected intrathecally into naive rats. Bilateral (L, left; R, right) mechanical hypersensitivity developed on the next day in rats receiving NGF 3 μg (C) or 10 μg (D), and did not appear in rats receiving vehicle (A) or NGF 1 μg (B). (E) When anti-NGF was intrathecally injected on day 4 after spinal nerve ligation, the contralateral mechanical hypersensitivity (mirror pain) was soon relieved on the next day. (F) Nonspecific IgG could not attenuate mirror pain. n = 6; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ for the comparison with the baseline (BL) on the corresponding side by Tukey's post hoc test after one-way analysis of variance.

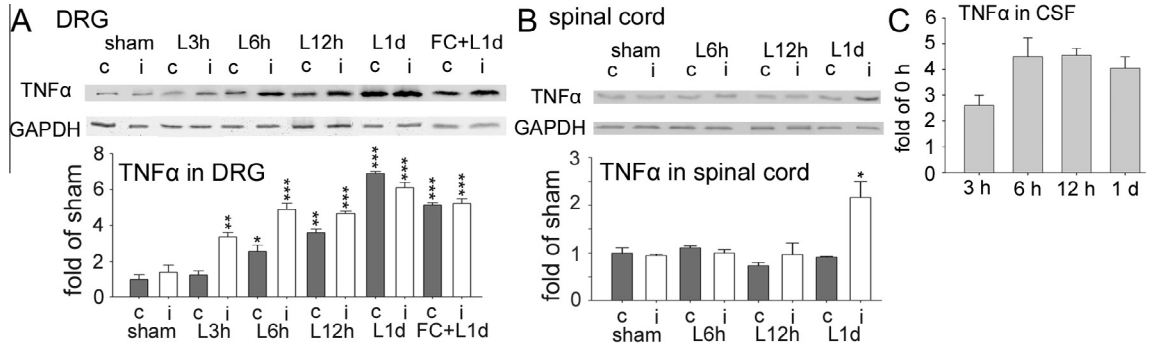

Fig. 8. Increase in tumor necrosis factor α (TNF-α) in the contralateral dorsal root ganglion (DRG) is later than in cerebrospinal fluid (CSF). Rats were sacrificed at 0 hours, 3 hours (L3h), 6 hours (L6h), 12 hours (L12), or 1 day (L1d) after spinal nerve ligation (SNL) or 1 day after sham operation. "FC+L1d" indicates intrathecal injection of fluorocitrate (FC) 30 minutes before ligation. (A–B) TNF-α levels in the contralateral (c) and ipsilateral (i) L5/L6 DRG (A) or dorsal spinal cord (B) were analyzed by western blot. n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test) for the comparison with the contralateral side of sham-operated rats. (C) TNF-α in CSF was measured by ELISA, and the control (0 hours for each rat immediately before SNL) was set as 1-fold. Note that the increase in TNF-α started at 3 hours in the ipsilateral DRG and CSF but at 6 hours in the contralateral DRG.

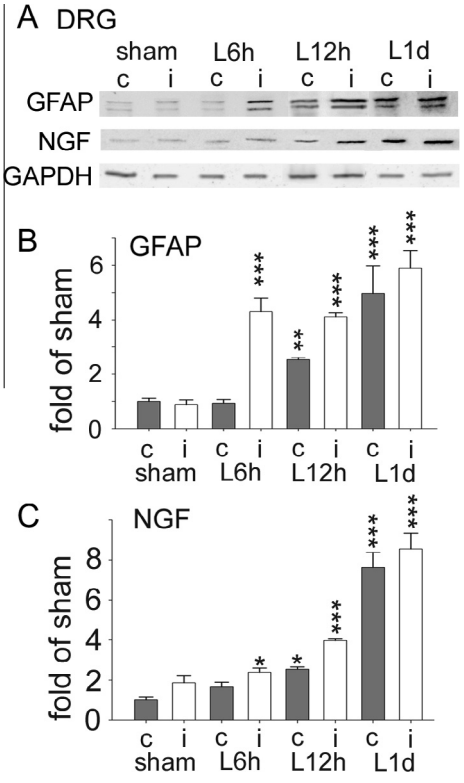

Fig. 9. Satellite glial activation correlates well with nerve growth factor (NGF) production in the dorsal root ganglion (DRG). (A) Western blot was used to analyze the levels of glial fibrillary acidic protein (GFAP; a marker of activated satellite glia) and NGF in the bilateral L5/L6 DRG at 6 hours (L6h), 12 hours (L12h), and 1 day (L1d) after SNL. (B, C) Quantitative data show that GFAP and NGF levels in the contralateral DRG were significantly increased 12 hours and 1 day after SNL. n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (Student's *t* test) for comparison with the contralateral side of sham-operated rats. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

At 6 hours after SNL, there was a 2-fold increase in NGF and a 5-fold increase in GFAP in the ipsilateral DRG (Fig. 9). It was not until 12 hours that the contralateral DRG showed evidence of an increase in NGF and GFAP (Fig. 9). In the contralateral DRG, because TNF-α was already increased at 6 hours (Fig. 8A), earlier than satellite glial activation and NGF production at 12 hours, these results suggest that satellite glia are activated by TNF-α to produce excess NGF.

3.11. Nonactivated satellite glia in the contralateral DRG express TNF-α receptor

We tested whether there are TNF-α receptors on nonactivated satellite glia to trap TNF-α. Antibodies against TNF receptor 1 (TNFR1), but not those against TNF receptor 2, have been shown to reduce mechanical hypersensitivity after peripheral nerve injury [47]. Furthermore, TNFR1 is upregulated in satellite glia of the ipsilateral DRG after sciatic nerve crush in mice [37]. We therefore examined TNFR1-IR in the contralateral DRG at 6 hours after SNL, when satellite glia had not been activated (Fig. 9B). By contrast with the few TNFR1(+) glia surrounding DRG neurons in sham-operated rats, there were many more TNFR1(+) satellite glia in the contralateral DRG of SNL rats (Fig. 10A). Weaker TNFR1-IR was detected in the somata of some contralateral DRG neurons (Fig. 10A). GFAP labels activated satellite glia, whereas glutamate aspartate transporter (GLAST) marks both nonactivated and activated satellite glia. Colocalization with GLAST confirmed the presence of TNFR1 on satellite glia (Fig. 10B). Satellite glia expressed GLAST but not GFAP at this time (Fig. 10C), indicating that they were not activated. Thus, nonactivated satellite glia in the contralateral DRG could be activated by TNF-α because of TNFR1 expression.

3.12. Exogenous TNF-α activates satellite glia to produce excess NGF

To investigate whether the excess NGF produced by activated satellite glia is induced by TNF-α, we intrathecally injected TNF-α and then immunostained for GFAP and NGF in the DRG. A previous study demonstrated that 20 ng TNF-α could induce mechanical and thermal hypersensitivity in naive mice at 3 hours after intrathecal injection [10]. Our data showed that 3 μg TNF-α could induce mechanical hypersensitivity in naive rats (Fig. 11A) but not thermal hypersensitivity (data not shown). Injection of 1 μg TNF-α did not evoke mechanical or thermal hypersensitivity (data not shown). The discrepancy might result from different species, drug doses, and behavioral testing times. Interestingly, when 10 μg anti-NGF was injected at 6 hours after TNF-α administration, mechanical hypersensitivity was relieved (Fig. 11B), indicating that NGF is the downstream effector of the TNF-α-triggered pain pathway. In the L5 DRG of rats receiving 3 μg TNF-α for 1 day, GFAP- and NGF-IR were greatly increased in glial cells surrounding the somata of DRG neurons (Fig. 11C, D). NGF-IR appeared only in GFAP(+) satellite glia (Fig. 11E), suggesting that NGF is produced by TNF-α-activated satellite glia. By contrast, there was no significant change of GFAP-IR in the L5 dorsal spinal

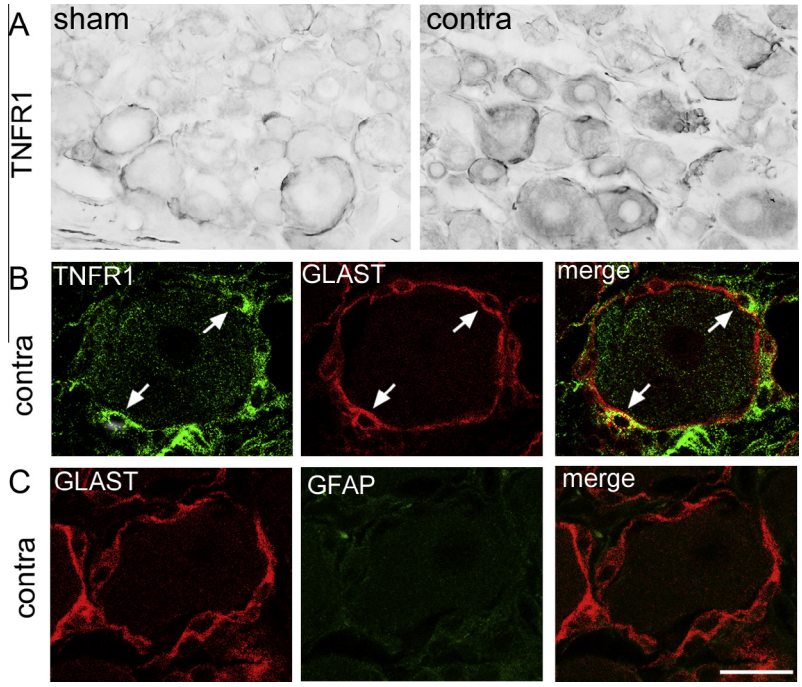

Fig. 10. Nonactivated satellite glia express tumor necrosis factor receptor 1 (TNFR1). Rats were sacrificed at 6 hours after sham operation or spinal nerve ligation (SNL), and sections of the contralateral L5 dorsal root ganglion (DRG) were immunostained. (A) There were many more TNFR1(+) glial cells surrounding DRG neurons in the SNL rats (right) than in the sham-operated rats (left). (B) Colocalization with glutamate aspartate transporter (GLAST) shows TNFR1 on satellite glia. (C) Satellite glia expressed GLAST but not glial fibrillary acidic protein (GFAP), indicating TNFR1 on nonactivated satellite glia. Scale bar (shown in C): 40 μm (A); 20 μm (B, C).

cord (Fig. 11F), showing that TNF-α did not induce astrocyte activation. SPD304, a small molecule, can interact with TNF-α and inhibit TNF-α binding to TNFR1 [15]. To confirm the initiative role of TNF-α in the induction of mirror pain, SPD304 was intrathecally injected into rats 30 minutes before SNL. One day later, we found that SPD304 not only abolished SNL-evoked mirror pain (Fig. 11G, H) but also suppressed SNL-induced satellite glial activation and NGF production in the contralateral L5 DRG (Fig. 11I, J). These data confirm that TNF-α can activate satellite glia to produce excess NGF in the DRG, which results in mechanical hypersensitivity.

4. Discussion

We present several lines of evidence to show that TNF-α-activated satellite glia in the contralateral DRG produce excess NGF to induce mirror pain after peripheral nerve injury. First, satellite glial activation was much faster than the macrophage response in the contralateral DRG, and DRG satellite glia were activated earlier than spinal astrocytes on the contralateral side (Figs. 1–3). Second, earlier and more dramatically than the other neurotrophic factors, NGF increased more quickly and on a larger scale in the DRG than in the dorsal spinal cord and hind paw skin on the contralateral side (Fig. 4). Third, the excess NGF produced by activated satellite glia greatly enhanced nociceptor excitability, which led to mechanical hypersensitivity in living animals (Figs. 5–7). Fourth, anti-NGF therapy effectively relieved nerve injury-evoked mirror pain and TNF-α-evoked mechanical hypersensitivity (Figs. 7 and 11). Fifth, TNF-α produced from the injured side after nerve injury, which diffused into the contralateral DRG via the CSF, rapidly activated satellite glia to produce excess NGF (Figs. 8–11). Since the DRG is located in the PNS, we propose a peripheral mechanism for the induction of mirror pain, which is summarized in Fig. 12.

4.1. Mirror pain is induced by DRG satellite glia but not by spinal astrocytes

Both DRG and spinal cord are located in the subarachnoid space and accessible to the CSF; however, cells within the spinal cord are further protected by the pia mater, the continuous sheath formed by subpial astrocytes, and the white matter [39]. Fluorescence-tagged oligonucleotide or protein was clearly detected in the DRG but barely detected in the spinal cord after intrathecal injection (see Supplementary Fig. 2) [3,5], and decreases in channel proteins have been observed in DRG cells by using antisense oligonucleotides [3,5], demonstrating that drugs function after uptake by DRG cells. Spatially, the bilateral L5 DRG are close to the junction between the L5 and L6 spinous processes (the site of intrathecal injection in this report), whereas the L5 spinal cord is near the junction between the T13 and L1 spinous processes [16]. One may argue that drug concentration in the CSF is higher near the injection site, such that drug concentration close to the L5 spinal cord is too low. L5 DRG cells could take up fluorescence-tagged oligonucleotide when it was applied via intrathecal cannulation and the opening was located between the L1 and L2 spinous processes [5], suggesting that drugs in the CSF can still diffuse into a DRG at a distance from the injection site. Thus, molecules administered by intrathecal routes can be taken up by DRG cells much more easily than by spinal cells.

Based simply on the observation that intrathecal injection of fluorocitrate (a glial inhibitor) can prevent inflammation-induced mirror pain at the behavioral level, but without examination of glial cells in spinal cord and DRG, it has been suggested that spinal astrocytes are necessary for mirror pain development [34]. In addition, because intrathecal infusion of propentofylline (another glial inhibitor) prevented nerve injury-evoked bilateral allodynia, with an examination of glial activation only in spinal cord, it has been concluded that spinal astrocytes contribute to the development of bilateral allodynia [36]. Since bilateral spinal astrocytes

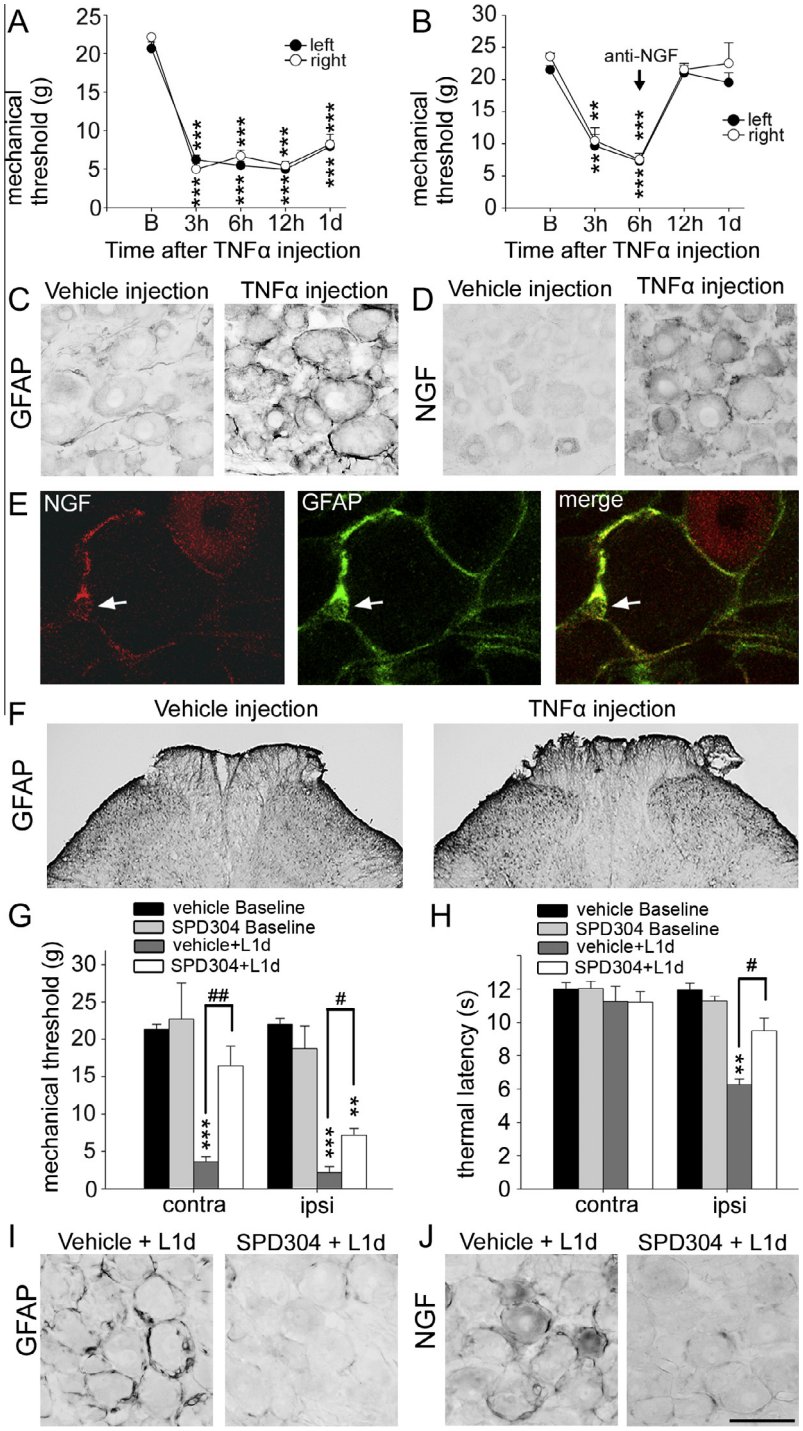

Fig. 11. Tumor necrosis factor α (TNF-α) activates satellite glia to produce excess nerve growth factor (NGF) in the dorsal root ganglion (DRG). (A) Rats developed mechanical hypersensitivity soon after intrathecal injection of 3 μg TNF-α. (B) Intrathecal injection of 10 μg anti-NGF 6 hours after TNF-α application soon relieved TNF-α-evoked mechanical hypersensitivity. (C–F) Rats were injected with vehicle or 3 μg TNF-α and sacrificed 1 day later. Sections of the L5 DRG and spinal cord were immunostained for glial fibrillary acidic protein (GFAP) and/or NGF. TNF-α induced satellite glial activation (C) and excess NGF production in the DRG (D), and double staining showed NGF expression in GFAP(+) activated satellite glia (arrow in E). There was no significant difference in GFAP immunoreactivity in the spinal cord between vehicle- and TNF-α-injected rats (F). (G–J) Rats were injected with TNF-α–TNFR1 binding inhibitor SPD304 30 minutes before spinal nerve ligation (SNL). One day later, SPD304 not only attenuated SNL-evoked bilateral mechanical hypersensitivity (G) and ipsilateral thermal hypersensitivity (H), but also suppressed SNL-induced satellite glial activation (I) and NGF production (J) in the contralateral L5 DRG. $^{**}P < 0.01$, $^{***}P < 0.001$ for the comparison with the baseline (B) for each group; $^{\#}P < 0.05$ for the comparison of the indicated pairs. Scale bar (shown in J): 66 μm (C, D, I, J); 20 μm (E); 300 μm (F).

remained activated during glial inhibitor infusion, and DRG glia were not examined in this report [36], the analgesic effect of propentofylline may result from the inhibition of DRG glia. Furthermore, the gap junction decoupler carbenoxolone attenuated mirror pain for 4 hours when injected intrathecally at day 10 after sciatic nerve injury [49], and spinal astrocytes were activated ipsilaterally at day 1 and bilaterally at day 7 after SNL (Fig. 2). These findings suggest that contralateral spinal astrocytes, which are activated by ipsilateral spinal astrocytes via gap junctions at a later stage after peripheral nerve injury, might be involved in the

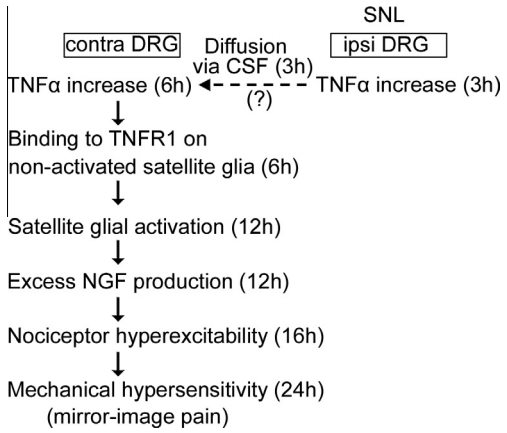

Fig. 12. Possible mechanism underlying peripheral nerve injury-evoked mirror pain. Following spinal nerve ligation (SNL) on the right side, tumor necrosis factor α (TNF-α) is increased greatly in the ipsilateral (ipsi) dorsal root ganglion (DRG), diffuses to the contralateral (contra) DRG via cerebrospinal fluid (CSF), and then activates satellite glia after binding to its receptor, TNFR1. Activated satellite glia produce excess nerve growth factor (NGF) which, in turn, enhances nociceptive excitability. Hyperexcitable nociceptors on the contralateral side evoke mirror pain in response to non-noxious mechanical stimuli. The number in each parenthesis indicates the time (in hours) after SNL.

maintenance of mirror pain. Therefore, it is unlikely that the spinal astrocyte is the major cell type involved in the induction of mirror pain.

Activated satellite glia in the DRG of an injured spinal nerve release excess NGF, TNF-α, or interleukin-1 to sensitize nociceptors, which contributes to the initiation of neuropathic pain [45,52], and the inhibition of satellite glia effectively alleviates SNL-evoked mechanical hypersensitivity [30]. Here, in animals with nerve injury-evoked mirror pain, we showed that activated satellite glia in the contralateral DRG behave similarly to those in the ipsilateral DRG. Inhibition of satellite glial activation by fluorocitrate pretreatment effectively delayed the onset of mirror pain (Fig. 1). There was rapid and dramatic activation of DRG satellite glia, but no significant changes in DRG Schwann cells/macrophages and spinal astrocytes/oligodendrocytes/microglia (Figs. 2 and 3). In particular, activated satellite glia in the contralateral DRG produced excess NGF, an effect that was abolished when satellite glial activation was inhibited (Figs. 4 and 5). These findings strongly support the assertion that satellite glia are the major cell type to induce mirror pain. Thus, activated satellite glia are required not only for the initiation of neuropathic pain on the injured side but also for the induction of mirror pain on the contralateral side.

4.2. NGF is the major mediator for the induction of mirror pain

Long-lasting mechanical hypersensitivity can be produced in rats by administering exogenous NGF via different routes, such as intraperitoneal injection daily for 4 days [28], single subcutaneous injection into a hind paw [12], or direct delivery to the DRG via a microcatheter inserted along the spinal nerve for 7 days [64]. In this report, long-lasting mechanical hypersensitivity in rats was evoked not only by a single intrathecal injection of exogenous NGF (Fig. 7C, D) but also by a very high level of endogenous NGF in the contralateral DRG at 1 day after unilateral nerve injury (Fig. 4D). Animals did not show mirror pain behavior when the high NGF level was prevented by a glial inhibitor (Figs. 1C, 4D), a TNF-α inhibitor (Fig. 11G, J), or directly neutralized by anti-NGF (Fig. 7E). All the data support a high NGF level in the contralateral DRG being sufficient to induce long-lasting mechanical hypersensitivity on the contralateral side (ie, mirror pain). Although the role of NGF in pain on the injured side has been extensively studied [33], we are the first to address NGF being the major mediator for the induction of mirror pain.

4.3. TNF-α triggers mirror pain by activating satellite glia to produce excess NGF

A report that spinal nerve crush-evoked mirror pain first appeared at day 2, followed by TNF-α upregulation/satellite glial activation in the contralateral DRG at day 7, suggested an association between them [14]. However, because mirror pain occurred earlier than TNF-α upregulation/satellite glial activation [14], this report could not explain how mirror pain is induced. Furthermore, although it has been shown that intraplantar TNF-α injection drives an increase in NGF in ipsilateral hind paw skin [58], the cell type producing NGF remains unclear. Here, we not only define the temporal correlation that mirror pain is induced after TNF-α upregulation/satellite glial activation in the contralateral DRG, but also identify satellite glia to be the type of cells that can produce excess NGF after activation by TNF-α.

Similar to NGF [17,19,23,53,61], a high TNF-α level can also excite nociceptors to evoke mechanical hypersensitivity [21, 43,56]. Different from NGF as the major mediator for the induction of mirror pain, TNF-α triggers the induction phase of mirror pain. SNL induced a series of changes in the contralateral DRG: TNF-α upregulation first, then satellite glial activation, and finally excess NGF production from activated satellite glia in the contralateral DRG (Figs. 8 and 9). Expression of TNFR1 on nonactivated satellite glia facilitates the activation of satellite glia by an increasing TNF-α level in the contralateral DRG soon after SNL (Figs. 10 and 11G–J), and activated satellite glia can produce excess NGF (Fig. 11C–E). In particular, anti-TNF-α could not relieve pain when it was applied after nerve injury [44,60], whereas anti-NGF effectively reversed TNF-α-evoked mechanical hypersensitivity (Fig. 11A, B). All these studies support NGF being the downstream effector of the TNF-α-triggered pain pathway.

4.4. Concluding remarks

Mirror pain accompanies many clinical pain states, such as complex regional pain syndrome [20], rheumatoid arthritis [32], and neuropathic pain [26]. Although mirror pain is usually not as severe as the pain on the injured side, patients still suffer on the contralateral side. In this report, to elucidate whether the central nervous system or PNS is involved in the induction of mirror pain after peripheral nerve injury, we examined some pathological changes in the bilateral spinal cord and DRG at early time points, quite different from the late time points analyzed in previous related studies. Within the first day after nerve injury we found the following sequential changes in the contralateral DRG: first TNF-α upregulation, followed by satellite glia activation, and finally increased NGF production (Fig. 12). By contrast, there was no significant astrocyte activation or microglial recruitment in the contralateral dorsal spinal cord. Because satellite glial activation is necessary for the induction of mirror pain, early treatment with reagents that inhibit satellite glial activation or their excess NGF production can effectively relieve mirror pain. Furthermore, since intrathecal application of small hydrophilic compounds or proteins can easily diffuse via the CSF into the DRG, but cannot permeate into the spinal cord [3,5, this report], the sensory ganglion is a potential target for pain therapy [42].

Conflict of interest statement

The authors declare no conflicts of interest in this study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2014.01.010.

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