Effects of Regional and Whole-body Hypothermic Treatment before and after Median Nerve Injury on Neuropathic Pain and Glial Activation in Rat Cuneate Nucleus

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ABSTRACT

Background: Neuroprotective effects of hypothermia on peripheral nerve injury remain uncertain. This study investigated the efficacy of hypothermia in attenuating neuropathic pain and glial activation in the cuneate nucleus in a median nerve chronic constriction injury (CCI) model.

Methods: Sprague-Dawley rats (n = 246) that underwent median nerve ligation at the elbow received various degrees of regional and whole-body hypothermia 15 min before CCI and 5 h, 1, 3, and 5 days after CCI. Hypothermia was maintained for 4 h. Seven days after CCI, behavioral and electrophysiological testings were conducted. Immunohistochemistry, immunoblotting, and enzyme-linked immunosorbent assay were used for qualitative and quantitative analysis of glial activation and measuring pro-inflammatory cytokines, respectively.

Results: Mild (32°C) and deep (28°C) regional hypothermia administered preinjury and 5 h postinjury attenuated neuropathic pain and glial activation. Application of whole-body hypothermia preinjury and 5 h postinjury provided a similar therapeutic effect. However, whole-body hypothermia, but not regional hypothermia, applied 1, 3, and 5 days postinjury attenuated glial activation and neuropathic pain. Similarly, on days 1, 3, and 5 postinjury, only whole-body hypothermia was effective in decreasing proinflammatory cytokine levels. The increase in injury discharge observed after CCI could be suppressed by regional or whole-body hypothermia at different stages of nerve injury.

Conclusions: At the early stage following nerve injury, regional and whole-body hypothermia reduces neural degeneration after peripheral nerve injury, but its effect on nerve injury-induced neuropathic pain has not been examined.

What We Already Know about This Topic
• Hypothermia reduces neural degeneration after peripheral nerve injury, but its effect on nerve injury-induced neuropathic pain has not been examined

What This Article Tells Us That Is New
• In rats, regional and whole body hypothermia at the time of nerve injury reduced behavioral signs of neuropathic hypersensitivity as well as concomitant glial activation in the cuneate nucleus
• These data suggest that regional or whole body hypothermia during surgery might reduce the incidence of chronic pain

EUROPATHIC pain afflicts millions of people worldwide and is one of the most significant health problems.1,2 Neuropathic pain may be associated with peripheral nerve injury, inflammation, or infection.3–5 Periph-
eral nerve injury evokes a barrage of injury discharges originating from the injured nerve fibers and their dorsal root ganglia; subsequently, these ectopic discharges facilitate the release of excitatory amino acids from damaged primary afferents, which cause central sensitization and contribute to the development of neuropathic pain.\(^6\)–\(^8\) Neuropathic pain manifests as spontaneous pain, allodynia (pain evoked by a normally innocuous stimulus), or hyperalgesia (enhanced pain evoked by a noxious stimulus). Tactile allodynia in particular is a cardinal symptom of neuropathic pain.\(^3\) Neuropathic pain can last for months or even years after the primary tissue damage has healed. Current treatments for this type of pain are often of limited efficacy and are beneficial in only a few patients.\(^3,9\) Therefore, developing effective treatments for neuropathic pain is an urgent matter.

Recently, hypothermia has been established as an effective neuroprotective treatment for peripheral nerve injury induced by multiple arterial ligatures. Several mechanisms of hypothermic neuroprotection have been proposed, including suppression of the inflammatory response,\(^10\) reduction of fiber degeneration and demyelination,\(^11–13\) and amelioration of motor dysfunction.\(^14\) Electrophysiological studies have demonstrated that peripheral nerve ischemia-induced sensory nerve dysfunction can be improved by hypothermic treatment.\(^11,12\) However, the effects of hypothermic treatment on preventing neuropathic pain have not yet been investigated.

Accumulating evidence suggests that following peripheral nerve injury, glia are involved in a cascade of events leading to the development of neuropathic pain.\(^15,16\) Further, the majority of human peripheral nerve injuries occur in the upper limbs, and median nerve injury is a common form of peripheral neuropathy caused by laceration, fracture-associated stretch and contusion, compression, and injection injuries.\(^17,18\) Accordingly, we have developed an experimental model of chronic constriction injury (CCI) on the median nerve based on the methods described by Bennett and Xie.\(^6\) Briefly, anesthesia was induced by an intraperitoneal injection of 7% chloral hydrate (0.45 ml/100 g body weight). Under a dissecting microscope, the right median nerve was separated from the surrounding tissue at the elbow level, immediately proximal to the entry between two heads of the pronator teres muscle. Four loose ligatures (4.0 chromic gut) were made around the peripheral nerve, and the incision was then sutured. For the sham surgeries, the median nerve was exposed in the same area of the right forelimb without ligation.

In this model, activation of microglia and astrocytes was observed in the cuneate nucleus (CN) after CCI of the median nerve.\(^19–22\) Activation of microglia and astrocytes in the CN is a hallmark of neuropathic pain.\(^23\) Electrode stimulation of the cuneate nucleus induces tactile allodynia in rats.\(^19,21\) In our model, for example, we observed activation of microglia in the CN after CCI of the median nerve.\(^19,21\) Immunohistochemical analysis revealed that microglial activation was increased in the CN after CCI of the median nerve.\(^19,21\) Furthermore, inhibition of microglial activation by minocycline prevented the development of neuropathic pain.\(^21\) Microinjection of the astrocytic toxin fluorocitrate also reversed nerve injury-induced mechanical allodynia.\(^19\) These data support an important role for microglia and astrocytes in the development of neuropathic pain.

In the present study, we aimed to evaluate the efficacy of hypothermia in relieving neuropathic pain and attenuating glial activation responses in a median nerve CCI model of neuropathic pain. We manipulated the degree of hypothermia (28°C or 32°C), initiation time of hypothermia (preinjury; 5 h, 1, 3, and 5 days postinjury), and the location of hypothermia application (regional or whole-body).

### Materials and Methods

#### Animal Preparation

These experiments were reviewed and approved by the National Science Council Committee as well as the Animal Center Committee at the College of Medicine of Fu Jen Catholic University, Taipei, Taiwan. The ethical guidelines of the International Association for the Study of Pain\(^23\) for animal experimentation were followed. All efforts were made to minimize animal suffering and reduce the number of experimental animals used. Animals used in this study were male Sprague-Dawley rats weighing 180–250 g, which were housed under approved conditions with a 12-h light-dark cycle and food and water available \textit{ad libitum}.

#### Nerve Injury Surgery

The experimental model of median nerve CCI was based on the methods described by Bennett and Xie.\(^6\) Briefly, anesthesia was induced by an intraperitoneal injection of 7% chloral hydrate (0.45 ml/100 g body weight). Under a dissecting microscope, the right median nerve was separated from the surrounding tissue at the elbow level, immediately proximal to the entry between two heads of the pronator teres muscle. Four loose ligatures (4.0 chromic gut) were made around the nerve,\(^19–22\) and the incision was then sutured. For the sham surgeries, the median nerve was exposed in the same area of the right forelimb without ligation.

#### Hypothermia Procedure

##### Regional Hypothermia

Under general anesthesia, a temperature therapy pad (Model TP3E; Gaymar Industries, Inc., Orchard Park, NY) was wrapped around the right forelimb to manipulate the temperature of the limb. The tubing of the temperature therapy pad was connected to a water-cooling circulator (Model CL-300R; TAITEC Co., Tokyo, Japan). The limb temperature was monitored by a digital electronic thermometer (Model TH-8; Physitemp Instruments, Inc., Clifton, NJ) with a 0.15-min diameter temperature probe (Model ICT-4; Physitemp Instruments, Inc.) attached to the surface of the pronator teres muscle. The temperature of the right forelimb was maintained at 37°C (normothermia), 32°C (mild hypothermia), or 28°C (deep hypothermia). Deep rectal temperature, measured by inserting a rectal probe (Model RET-2; Physitemp Instruments, Inc.) 7 cm into the rectum, was also monitored, and was maintained at 37°C using a servo-controlled infrared lamp. The target temperature was achieved within 15 min.

The experimental animals \((n = 123)\) were divided into six groups according to the timing of induction of regional hypothermia. In the first group (preinjury group), the temperature of the right forelimb was maintained at 37°C \((n = 7)\), 32°C \((n = 7)\), or 28°C \((n = 7)\) 15 min before ligation of the median nerve. In the second group \((5 \text{ h postinjury group})\), the temperature of the right forelimb was maintained at 37°C \((n = 7)\), 32°C \((n = 7)\), or 28°C \((n = 7)\) 5 h after

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median nerve injury. In the third group (1 day postinjury group), the temperature of the right forelimb was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 1 day after median nerve injury. In the fourth group (3 days postinjury group), the temperature of the right forelimb was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 3 days after median nerve injury. In the fifth group (5 days postinjury group), the temperature of the right forelimb was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 5 days after median nerve injury. In the sixth group (sham-operated group), the whole-body temperature was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 1 day after the sham operation (median nerve exposure without ligation). The temperature of the right forelimb was maintained at the target temperature for 4 h in all rats.

Whole-body Hypothermia. Under general anesthesia, the temperature therapy pad (Model TP22C; Gaymar Industries, Inc.) was wrapped around the whole body of the animal, and the tubing of the temperature therapy pad was connected to a water-cooling circulator. Rectal temperature was monitored using a digital electronic thermometer with a rectal probe inserted into the rectum. The rat’s rectal temperature was maintained at 37°C, 32°C, or 28°C.

The experimental animals (n = 123) were divided into six groups according to the timing of induction of whole-body hypothermia. In the first group (preinjury group), the whole-body temperature was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 15 min before ligation of the median nerve. In the second group (5 h postinjury group), the whole-body temperature was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 5 h after median nerve injury. In the third group (1 day postinjury group), the whole-body temperature was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 1 day after median nerve injury. In the fourth group (3 days postinjury group), the whole-body temperature was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 3 days after median nerve injury. In the fifth group (5 days postinjury group), the whole-body temperature was maintained at 37°C (n = 7), 32°C (n = 7), or 28°C (n = 7) 5 days after median nerve injury. In the sixth group (sham-operated group), the whole-body temperature was maintained at 37°C (n = 6), 32°C (n = 6), or 28°C (n = 6) 1 day after sham operation. The whole-body temperature was maintained at the target temperature for 4 h in all rats.

After regional or whole-body hypothermic treatment, rats were returned to their home cages, and the limb or body temperature was allowed to rise back to normal naturally. The animals survived for 7 days after median nerve injury, and then the behavioral testing and electrophysiological studies were conducted on these animals. Afterward, the rats were randomly assigned to one of the two experiments. In one experiment, the animals were processed for immunohistochemistry, and in the other experiment, the animals were processed for Western blotting and enzyme-linked immunosorbent assay.

Behavioral Testing

Mechanical Allodynia. Rats were placed in individual plexiglass chambers (25 × 40 × 18 cm) with wire mesh bottoms and were allowed to acclimatize to the environment for 30 min. The mechanical withdrawal threshold of the rat forepaw was determined using a series of von Frey filaments (Semmes-Weinstein Monofilaments, North Coast Medical, Inc., Gilroy, CA; bending force: 0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4.0, 6.0, 8.0, 10.0, 15.0, and 26.0 g). The measurement was performed according to the method described by Tal and Bennett.24 Quantitative mechanical stimuli were applied to the medial plantar surface of each forepaw in an ascending order to evaluate the withdrawal threshold. Each von Frey filament was applied five times. When rats showed at least two withdrawal responses to a filament, the bending force of the filament was defined as the withdrawal threshold.

Thermal Hyperalgesia. To measure the thermal withdrawal latency of the forepaws, the plantar test (Ugo Basile, Comerio, Italy) was utilized. Briefly, rats were individually placed in one of the three plexiglass containers (22 × 17 × 14 cm) located on the increased floor of a clear glass plate (3 mm thick) and allowed to habituate to the apparatus for 30 min. A radiant heat source was positioned under the glass plate directly beneath the plantar surface of the forepaw. The withdrawal latency was automatically measured as the time elapsed from the onset of radiant heat stimulation to the withdrawal of the forepaw. In order to avoid tissue damage, the maximum thermal stimulus duration was 20 s. A more detailed description of the apparatus has been published previously.25 Each forepaw was alternately tested five times with a minimal interval of 10 min between measurements, and readings were recorded to the nearest 0.1 s. Five latency values per side were averaged. Animals were tested at least 5 h after von Frey filament testing.

All studies were performed in a randomized, blinded manner to avoid expectation bias. In brief, all animals were selected in a random order for behavioral testing, and another investigator, who was blinded to the experimental status of the rats, made the observations.

Electrophysiology

Two hours after the end of behavioral testing, all animals were reanesthetized, and the right median nerve was carefully reexposed and dissected free. The nerve was placed on a pair of platinum hook electrodes just proximal to the site of nerve ligation or at the same site in sham-operated rats to record discharge activity of the nerve. Paraffin oil was applied around the exposed segment of the nerve to prevent drying. The electrodes were connected to the Xction View II Data Acquisition System (Model XD-04 II; Singa, Taoyuan, Taiwan), and the discharge signals were amplified 2000-fold,
recorded for 10 min, and analyzed using Xction View II software by an investigator blinded to the treatment group.

**Immunohistochemistry**

The rats were deeply anesthetized and perfused with 4% paraformaldehyde per 0.1 M phosphate buffer, pH 7.4. Medulla tissue blocks that contained the CN were resected and stored overnight in phosphate buffer with 30% sucrose, and 30-μm slices were transversely cut with a cryostat (Leica, Nussloch, Germany).

The immunohistochemical procedure was performed as described previously. In brief, floating medulla sections were collected and treated with 1% H2O2, blocked with 2% normal goat serum (GibcoBRL, Gel Company, San Francisco, CA), and incubated in either mouse monoclonal anti-OX-42 (CD11b, 1:200; Serotec, Indianapolis, IN) or mouse monoclonal anti-glial fibrillary acidic protein (GFAP) (1:800; Sigma-Aldrich, St. Louis, MO) antibody for 48 h at 4°C. After rinsing with phosphate-buffered saline, the sections were incubated in 1:200 biotinylated antirat IgG (Vector, Burlingame, CA), and processed with avidin-biotin-peroxidase complex (Vector). Peroxidase activity was subsequently visualized by treating with Vector SG Substrate Kit. Finally, the floating sections were mounted onto gelatinized slides and examined under a light microscope (Zeiss Axiohot, Jena, Germany). For negative controls, in which immunolabeling was not present, sections were incubated in normal mouse serum in lieu of the above-mentioned primary antibodies, or with the omission of the same antibodies.

**Sample Preparation and Western Blot Analysis**

After rats were sacrificed by decapitation, the CN was micro-dissected according to the method described by Palkovits and Brownstein. The punched tissue was homogenized in 100 μl lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM Na3VO4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 20 μg/ml pepstatin A) with a grinder on ice. The homogenate was centrifuged at 10,000 g for 20 min at 4°C. The supernatant was collected and the levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were quantified using TNF-α Immunoassay Kit (R&D Systems, Inc., Minneapolis, MN) and IL-1β Immunoassay Kit (R&D Systems, Inc.), respectively. All procedures were performed following manufacturer’s instructions; microplates were read using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

**Enzyme-linked Immunosorbent Assay**

The concentration of proinflammatory cytokines in the CN was determined by the enzyme-linked immunosorbent assay. The sample was prepared as described above. The supernatant was collected and the levels of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were quantified using TNF-α Immunoassay Kit (R&D Systems, Inc., Minneapolis, MN) and IL-1β Immunoassay Kit (R&D Systems, Inc.), respectively. All procedures were performed following manufacturer’s instructions; microplates were read using a microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

**Data Presentation and Statistical Analysis**

For Western blot analysis, the optical density of specific OX-42 and GFAP bands was measured with a computer-assisted image analysis system (Gel-Pro Analyzer software, Media Cybernetics, Inc., Bethesda, MD). All results are presented as the mean ± SD. Mean values were analyzed using two-way ANOVA followed by Tukey post hoc tests. There were no missing data needing adjustment. All P values were two-tailed and a P value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL).

**Results**

**Effect of Regional or Whole-body Hypothermic Treatment on Behavior on Day 7 after Median Nerve CCI**

Behavioral testing showed that sham-operated rats treated with regional normothermia (37°C), mild regional hypothermia (32°C), or deep regional hypothermia (28°C) had similar mechanical withdrawal thresholds and thermal withdrawal latencies (fig. 1A-B). In CCI rats pretreated with regional normothermia, the mechanical withdrawal threshold and thermal withdrawal latency were significantly decreased compared with sham-operated rats (figs. 1A and B). However, a significant increase in the mechanical withdrawal threshold and thermal withdrawal latency was observed in CCI rats pretreated with mild or deep regional hypothermia compared with rats pretreated with regional normothermia (figs. 1A and B). Similarly, in the 5 h postinjury group, there was a significant increase in the mechanical withdrawal threshold and thermal withdrawal latency in CCI rats treated with mild or deep regional hypothermia compared with those treated with regional normothermia (figs. 1A and B). In the 1 day, 3 days, and 5 days postinjury groups, there were...
no differences in mechanical withdrawal threshold and thermal withdrawal latency among CCI rats treated with regional normothermia, mild regional hypothermia, and deep regional hypothermia (figs. 1A and B). Furthermore, in the preinjury and 5 h postinjury groups, mechanical allodynia (A) and thermal hyperalgesia (B) were significantly suppressed in chronic constriction injury (CCI) rats treated with mild or deep regional hypothermia compared with those treated with regional normothermia (*P < 0.05, by Tukey test). In addition, deep regional hypothermia administered preinjury and 5 h postinjury was more effective in ameliorating mechanical allodynia (A) and thermal hyperalgesia (B) than mild regional hypothermia (*P < 0.05, by Tukey test). In 1 day, 3 days, and 5 days postinjury groups, there were no significant differences in the mechanical withdrawal threshold (A) and thermal withdrawal latency (B) among CCI rats treated with regional normothermia, mild regional hypothermia, or deep regional hypothermia (P > 0.05, by Tukey test). Similarly, a significant improvement in both mechanical allodynia (C) and thermal hyperalgesia (D) was achieved after applying whole-body hypothermia (*P < 0.05, by two-way ANOVA). In preinjury, 5 h, 1 day, and 3 days postinjury groups, mechanical allodynia (C) and thermal hyperalgesia (D) were significantly suppressed in CCI rats treated with mild or deep whole-body hypothermia compared with those treated with whole-body normothermia (*P < 0.05, by Tukey test). In the 5 days postinjury group, deep whole-body hypothermia, but not mild whole-body hypothermia, significantly attenuated mechanical allodynia (C) and thermal hyperalgesia (D) compared with whole-body normothermia (*P < 0.05, by Tukey test). In all five groups, deep whole-body hypothermia was more effective in ameliorating mechanical allodynia (C) and thermal hyperalgesia (D) than mild whole-body hypothermia (*P < 0.05, by Tukey test). Regional and whole-body normothermia had similar effects on mechanical allodynia and thermal hyperalgesia (P > 0.05, by two-way ANOVA). In contrast, mild and deep whole-body hypothermia were more effective in attenuating mechanical allodynia and thermal hyperalgesia than mild and deep regional hypothermia, respectively (P < 0.05, by two-way ANOVA). Error bars represent mean ± SD; n = 6 or 7 rats per treatment.
Effect of Hypothermia on Neuropathic Pain

Electrophysiological studies showed that sham-operated rats treated with regional normothermia displayed low-frequency spikes (fig. 2Aa). In CCI rats pretreated with regional normothermia, the rate of discharge was dramatically increased compared with that in sham-operated rats (figs. 2Aa and Ab). However, a significantly lower nerve discharge rate was observed in CCI rats pretreated with mild or deep regional hypothermia than in those pretreated with regional normothermia (figs. 2Ac and Ad).

Quantitative analysis revealed that low rates of nerve discharge were observed in sham-operated rats treated with regional normothermia, mild regional hypothermia, mild regional hypothermia, or deep regional hypothermia (fig. 2B). In the preinjury, 5 h, 1 day, and 3 days postinjury groups of CCI rats, the rate of discharge was significantly decreased in those treated with either mild or deep regional hypothermia compared with those treated with regional normothermia. In the 5 days postinjury group, the rate of nerve discharge was significantly decreased in CCI rats receiving deep, but not mild, regional hypothermia compared with those receiving regional normothermia. In addition, a significantly lower nerve discharge rate was observed in CCI rats that underwent deep regional hypothermia than in those that underwent mild regional hypothermia.

Low rates of nerve discharge were observed in sham-operated rats treated with whole-body normothermia, mild whole-body hypothermia, or deep whole-body hypothermia (fig. 2C). In the preinjury, 5 h, 1 day, and 3 days postinjury groups of CCI rats, the discharge rate was significantly decreased in those treated with either mild or deep whole-body hypothermia compared with those treated with whole-body normothermia. In the 5 days postinjury group, the rate of nerve discharge was significantly decreased in CCI rats receiving deep whole-body hypothermia, but not mild whole-body hypothermia, compared with those receiving whole-body normothermia. Moreover, the rate of discharge was significantly lower in CCI rats that received deep whole-body hypo-

Fig. 2. Effects of regional or whole-body hypothermic treatment on the discharge activity of the injured median nerve. A low frequency of spikes was observed in sham-operated rats treated with regional normothermia (Aa). In contrast, high rates of nerve discharge were seen in chronic constriction injury (CCI) rats pretreated with regional normothermia (Ab). In CCI rats pretreated with mild (Ac) or deep regional hypothermia (Ad), the discharge rate was decreased. (B) A significant decrease in the rate of nerve discharge was noted after application of regional hypothermia (*P < 0.05, by two-way ANOVA). In preinjury, 5 h, 1 day, and 3 days postinjury groups, the discharge rates were significantly decreased in CCI rats treated with mild or deep regional hypothermia compared with those treated with regional normothermia (*P < 0.05, by Tukey test). On postinjury day 5, deep, but not mild, regional hypothermia significantly decreased the rate of nerve discharge compared with regional normothermia (*P < 0.05, by Tukey test). Further, the discharge rate was significantly lower in CCI rats that received deep regional hypothermia than mild regional hypothermia (*P < 0.05, by Tukey test). (C) A significant decrease in the rate of nerve discharge was achieved after applying whole-body hypothermia (P < 0.05, by two-way ANOVA). The discharge rate was significantly decreased in CCI rats treated with mild or deep whole-body hypothermia compared with whole-body normothermia in preinjury, 5 h, 1 day, and 3 days postinjury groups (*P < 0.05, by Tukey test). Deep whole-body hypothermia, but not mild whole-body hypothermia, significantly decreased the rate of discharge compared with whole-body normothermia on postinjury day 5 (*P < 0.05, by Tukey test). In addition, the discharge activity was significantly lower in CCI rats that received deep whole-body hypothermia than in those that received mild whole-body hypothermia (P < 0.05, by Tukey test). Regional and whole-body hypothermia, either mild or deep, had similar effects on the nerve discharge rate (P > 0.05, by two-way ANOVA). Error bars represent mean ± SD; n = 6 or 7 rats per treatment.

A more profound increase in mechanical withdrawal threshold and thermal withdrawal latency was observed in CCI rats that were administered deep whole-body hypothermia compared with deep regional hypothermia (fig. 1). Similar results were also obtained when mild regional and whole-body hypothermia were applied.

Effect of Regional or Whole-Body Hypothemic Treatment on Injury Discharge on Day 7 after Median Nerve CCI

Electrophysiological studies showed that sham-operated rats treated with regional normothermia displayed low-frequency spikes (fig. 2Aa). In CCI rats pretreated with regional normothermia, the rate of discharge was dramatically increased compared with that in sham-operated rats (figs. 2Aa and Ab). However, a significantly lower nerve discharge rate was observed in CCI rats pretreated with mild or deep regional hypothermia than in those pretreated with regional normothermia (figs. 2Ac and Ad).

Quantitative analysis revealed that low rates of nerve discharge were observed in sham-operated rats treated with regional normothermia, mild regional hypothermia, deep regional hypothermia, or deep regional hypothermia (fig. 2B). In the preinjury, 5 h, 1 day, and 3 days postinjury groups of CCI rats, the rate of discharge was significantly decreased in those treated with either mild or deep regional hypothermia compared with those treated with regional normothermia. In the 5 days postinjury group, the rate of nerve discharge was significantly decreased in CCI rats receiving deep, but not mild, regional hypothermia compared with those receiving regional normothermia. In addition, a significantly lower nerve discharge rate was observed in CCI rats that underwent deep regional hypothermia than in those that underwent mild regional hypothermia.

Low rates of nerve discharge were observed in sham-operated rats treated with whole-body normothermia, mild whole-body hypothermia, or deep whole-body hypothermia (fig. 2C). In the preinjury, 5 h, 1 day, and 3 days postinjury groups of CCI rats, the discharge rate was significantly decreased in those treated with either mild or deep whole-body hypothermia compared with those treated with whole-body normothermia. In the 5 days postinjury group, the rate of nerve discharge was significantly decreased in CCI rats receiving deep whole-body hypothermia, but not mild whole-body hypothermia, compared with those receiving whole-body normothermia. Moreover, the rate of discharge was significantly lower in CCI rats that received deep whole-body hypo-

Anesthesiology 2012; 116:415–31
thermia than in those that received mild whole-body hypothermia.

When comparing between regional and whole-body hypothermia, there were no differences in nerve discharge rates with respect to each treatment temperature (figs. 2B and C).

Effect of Regional or Whole-body Hypothermic Treatment on Glial Activation on Day 7 after Median Nerve CCI

Microglial Activation Responses. Little OX-42 immunoreactivity was observed in the CN of sham-operated rats treated with regional normothermia (A), mild regional hypothermia (B), or deep regional hypothermia (C). In the preinjury and 5 h postinjury groups, OX-42 immunoreactivity decreased in the CN of CCI rats that received mild regional hypothermia (E, H) or deep regional hypothermia (F, I), but not in those that received regional normothermia (D, G). In 1 day, 3 days, and 5 days postinjury groups, there was intense OX-42 immunoreactivity in the CN of CCI rats treated with regional normothermia (J, M, P), mild regional hypothermia (K, N, Q), and deep regional hypothermia (L, O, R). Bar = 100 μm.
comparison with those treated with regional normothermia (figs. 3G–I). In the 1 day, 3 days, and 5 days postinjury groups, intense OX-42 immunoreactivity was observed in the ipsilateral CN of CCI rats that received regional normothermia, mild regional hypothermia, or deep regional hypothermia (C). In preinjury, 5 h, 1 day, and 3 days postinjury groups, OX-42 immunoreactivity was decreased in the CN of CCI rats that received mild (E, H, K, N) or deep whole-body hypothermia (F, I, L, O) compared with those that received whole-body normothermia (D, G, J, M). In the 5 days postinjury group, OX-42 immunoreactivity was decreased in the CN of CCI rats receiving deep whole-body hypothermia (R), but not in those receiving whole-body normothermia (P) or mild whole-body hypothermia (Q). Bar = 100 μm.

Fig. 4. Photomicrographs showing OX-42 immunoreactivity in the ipsilateral cuneate nucleus (CN) on day 7 after chronic constriction injury (CCI) in rats that received whole-body hypothermic treatment. There was little OX-42 immunoreactivity present in the CN of sham-operated rats treated with whole-body normothermia (A), mild whole-body hypothermia (B), or deep whole-body hypothermia (C). In preinjury, 5 h, 1 day, and 3 days postinjury groups, OX-42 immunoreactivity was decreased in the CN of CCI rats that received mild (E, H, K, N) or deep whole-body hypothermia (F, I, L, O) compared with those that received whole-body normothermia (D, G, J, M). In the 5 days postinjury group, OX-42 immunoreactivity was decreased in the CN of CCI rats receiving deep whole-body hypothermia (R), but not in those receiving whole-body normothermia (P) or mild whole-body hypothermia (Q). Bar = 100 μm.
whole-body hypothermia, but not mild whole-body hypothermia, compared with those receiving whole-body normothermia (figs. 4P–R).

Western blot analysis showed a significant decrease in the levels of OX-42 in the CN of CCI rats pretreated with mild or deep regional hypothermia compared with those pretreated with regional normothermia ($P < 0.05$, by Tukey test). Similarly, in the 5 h postinjury group, OX-42 levels in the CN were significantly decreased in CCI rats that received mild or deep regional hypothermia compared with those that received regional normothermia ($P < 0.05$, by Tukey test). Furthermore, when administered preinjury and 5 h postinjury, deep regional hypothermia was more effective in suppressing OX-42 levels than mild regional hypothermia ($P < 0.05$, by Tukey test). However, in 1 day, 3 days, and 5 days postinjury groups, the levels of OX-42 in the CN did not differ significantly among rats that received regional normothermia, mild regional hypothermia, or deep regional hypothermia ($P > 0.05$, by Tukey test). Moreover, in all five groups, lower levels of OX-42 were observed in CCI rats treated with deep whole-body hypothermia than in those treated with mild whole-body hypothermia ($P < 0.05$, by Tukey test). Regional and whole-body normothermia resulted in a similar increase in OX-42 levels ($P > 0.05$, by two-way ANOVA). Whole-body hypothermia, either mild or deep, contributed to lower OX-42 levels than the corresponding regional hypothermia ($P < 0.05$, by two-way ANOVA).

**Astrocyte Activation Responses.** Little immunoreactivity for GFAP was observed in the CN of sham-operated rats (fig. 6A). In the CCI rats, GFAP levels were significantly increased in rats treated with regional or whole-body hypothermia compared with those treated with regional normothermia (fig. 6B). Similarly, in the 5 h postinjury group, GFAP levels in the CN were significantly increased in CCI rats that received mild or deep regional hypothermia compared with those that received regional normothermia (fig. 6B). Furthermore, when administered preinjury and 5 h postinjury, deep regional hypothermia was more effective in suppressing GFAP levels than mild regional hypothermia (fig. 6B). However, in 1 day, 3 days, and 5 days postinjury groups, the levels of GFAP in the CN did not differ significantly among rats that received regional normothermia, mild regional hypothermia, or deep regional hypothermia (fig. 6B). Moreover, in all five groups, lower levels of GFAP were observed in rats treated with deep whole-body hypothermia than in those treated with mild whole-body hypothermia (fig. 6B). Regional and whole-body normothermia resulted in a similar increase in GFAP levels ($P > 0.05$, by two-way ANOVA). Whole-body hypothermia, either mild or deep, contributed to lower GFAP levels than the corresponding regional hypothermia ($P < 0.05$, by two-way ANOVA). $\beta$-actin was used as a loading control. Error bars represent mean ± SD; $n = 5$ rats per treatment.
treated with regional normothermia, mild regional hypothermia, or deep regional hypothermia (figs. 6A–C). In contrast, intense GFAP immunoreactivity was present in the ipsilateral CN of CCI rats pretreated with regional normothermia (A), mild regional hypothermia (B), or deep regional hypothermia (C). In preinjury and 5 h postinjury groups, GFAP immunoreactivity in the CN was decreased in CCI rats that received mild (E, H) or deep regional hypothermia (F, I) compared with those that received regional normothermia (D, G). In 1 day, 3 days, and 5 days postinjury groups, intense GFAP immunoreactivity was observed in the CN of CCI rats treated with regional normothermia (J, M, P), mild regional hypothermia (K, N, Q), or deep regional hypothermia (L, O, R). Bar = 100 μm.

Fig. 6. Photomicrographs showing glial fibrillary acidic protein (GFAP) immunoreactivity in the ipsilateral cuneate nucleus (CN) on day 7 days after chronic constriction injury (CCI) in rats that received regional hypothermic treatment. Little GFAP immunoreactivity was observed in the CN of sham-operated rats treated with regional normothermia (A), mild regional hypothermia (B), or deep regional hypothermia (C). In preinjury and 5 h postinjury groups, GFAP immunoreactivity in the CN was decreased in CCI rats that received mild (E, H) or deep regional hypothermia (F, I) compared with those that received regional normothermia (D, G). In 1 day, 3 days, and 5 days postinjury groups, intense GFAP immunoreactivity was observed in the CN of CCI rats treated with regional normothermia (J, M, P), mild regional hypothermia (K, N, Q), or deep regional hypothermia (L, O, R).

Little GFAP immunoreactivity was present in the CN of sham-operated rats treated with whole-body normothermia, mild whole-body hypothermia, or deep whole-body hypothermia (figs. 7A–C). In the preinjury group, intense GFAP immunoreactivity was observed in the ipsilateral CN of CCI rats pretreated with whole-body normothermia (fig. 7D). In
contrast, GFAP immunoreactivity in the ipsilateral CN of CCI rats pretreated with either mild or deep whole-body hypothermia was significantly decreased in comparison with CCI rats pretreated with whole-body normothermia (B), or deep whole-body hypothermia (C). In preinjury, 5 h, 1 day, and 3 days postinjury groups, GFAP immunoreactivity in the CN of CCI rats that received mild (E, H, K, N) or deep whole-body hypothermia (F, I, L, O) was decreased compared with those that received whole-body normothermia (D, G, J, M). On day 5 postinjury, GFAP immunoreactivity was decreased in the CN of CCI rats receiving deep whole-body hypothermia (R), but not in those receiving whole-body normothermia (P) or mild whole-body hypothermia (Q). Bar = 100 μm.

Fig. 7. Photomicrographs showing glial fibrillary acidic protein (GFAP) immunoreactivity in the ipsilateral cuneate nucleus (CN) 7 days after chronic constriction injury (CCI) in rats that received whole-body hypothermic treatment. Little GFAP immunoreactivity was present in the CN of sham-operated rats treated with whole-body normothermia (A), mild whole-body hypothermia (B), or deep whole-body hypothermia (C). In preinjury, 5 h, 1 day, and 3 days postinjury groups, GFAP immunoreactivity in the CN of CCI rats that received mild (E, H, K, N) or deep whole-body hypothermia (F, I, L, O) was decreased compared with those that received whole-body normothermia (D, G, J, M). On day 5 postinjury, GFAP immunoreactivity was decreased in the CN of CCI rats receiving deep whole-body hypothermia (R), but not in those receiving whole-body normothermia (P) or mild whole-body hypothermia (Q). Bar = 100 μm.

Western blot analysis showed a significant decrease in the levels of GFAP in the CN of CCI rats pretreated with either mild or deep regional hypothermia compared with those pretreated with regional normothermia (fig. 8A). Similarly, in the 5 h postinjury group, mild or deep regional hypothermia, but not regional normothermia, attenuated GFAP levels in the CN of CCI rats (fig. 8A). In the 1 day, 3 days, and

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5 days postinjury groups, neither regional normothermia nor regional hypothermia was effective in decreasing GFAP levels (fig. 8A). Furthermore, in both preinjury and 5 h postinjury groups, deep regional hypothermia compared with those treated with regional normothermia (*P < 0.05, by Tukey test). In the 5 h postinjury group, GFAP levels in the CN were also significantly decreased in CCI rats treated with mild or deep regional hypothermia compared with those treated with regional normothermia (*P < 0.05, by Tukey test). Furthermore, when administered preinjury and 5 h postinjury, deep regional hypothermia was more effective in suppressing GFAP levels than mild regional hypothermia (*P < 0.05, by Tukey test). However, in 1 day, 3 days, and 5 days postinjury groups, no significant differences in the levels of GFAP in the CN were observed among rats that received regional normothermia, mild regional hypothermia, or deep regional hypothermia (P > 0.05, by Tukey test). (B) A significant decrease in GFAP levels was noted after applying whole-body hypothermia (*P < 0.05, by two-way ANOVA). In preinjury, 5 h, 1 day and 3 days postinjury groups, mild and deep whole-body hypothermia were effective in decreasing GFAP levels in the CN of CCI rats compared with whole-body normothermia (*P < 0.05, by Tukey test). On day 5 postinjury, deep whole-body hypothermia, but not mild whole-body hypothermia, resulted in a significant decrease in the levels of GFAP compared with whole-body normothermia (*P < 0.05, by Tukey test). Whole-body hypothermia, either mild or deep, resulted in lower GFAP levels than the corresponding regional hypothermia (P < 0.05, by two-way ANOVA). Regional and whole-body normothermia contributed to a similar increase in GFAP levels (P > 0.05, by two-way ANOVA). β-actin was used as a loading control. Error bars represent mean ± SD; n = 5 rats per treatment.

Fig. 8. Western blot analysis showing the levels of glial fibrillary acidic protein (GFAP) in the ipsilateral cuneate nucleus (CN) on day 7 after chronic constriction injury (CCI) in rats treated with regional or whole-body hypothermia. (A) Application of regional hypothermia led to a significant decrease in GFAP levels (P < 0.05, by two-way ANOVA). In rats pretreated with mild or deep regional hypothermia, there was a significant decrease in GFAP levels compared with those pretreated with regional normothermia (*P < 0.05, by Tukey test). In the 5 h postinjury group, GFAP levels in the CN were also significantly decreased in CCI rats treated with mild or deep regional hypothermia compared with those treated with regional normothermia (*P < 0.05, by Tukey test). Furthermore, when administered preinjury and 5 h postinjury, deep regional hypothermia was more effective in suppressing GFAP levels than mild regional hypothermia (*P < 0.05, by Tukey test). However, in 1 day, 3 days, and 5 days postinjury groups, no significant differences in the levels of GFAP in the CN were observed among rats that received regional normothermia, mild regional hypothermia, or deep regional hypothermia (P > 0.05, by Tukey test). (B) A significant decrease in GFAP levels was noted after applying whole-body hypothermia (*P < 0.05, by two-way ANOVA). In preinjury, 5 h, 1 day and 3 days postinjury groups, mild and deep whole-body hypothermia were effective in decreasing GFAP levels in the CN of CCI rats compared with whole-body normothermia (*P < 0.05, by Tukey test). On day 5 postinjury, deep whole-body hypothermia, but not mild whole-body hypothermia, resulted in a significant decrease in the levels of GFAP compared with whole-body normothermia (*P < 0.05, by Tukey test). Whole-body hypothermia, either mild or deep, resulted in lower GFAP levels than the corresponding regional hypothermia (P < 0.05, by two-way ANOVA). Regional and whole-body normothermia contributed to a similar increase in GFAP levels (P > 0.05, by two-way ANOVA). β-actin was used as a loading control. Error bars represent mean ± SD; n = 5 rats per treatment.

Effect of Regional or Whole-body Hypothermic Treatment on Proinflammatory Cytokines on Day 7 after Median Nerve CCI

The CN of sham-operated rats treated with regional normothermia, mild regional hypothermia, or deep regional hypothermia showed low levels of TNF-α and IL-1β (figs. 9A and B). The levels of TNF-α and IL-1β in the ipsilateral CN of CCI rats pretreated with regional normothermia were markedly increased compared with those of sham-operated rats (figs. 9A and B). However, TNF-α and IL-1β levels in the ipsilateral CN of CCI rats pretreated with mild or deep regional hypothermia showed significant decreases compared with those pretreated with regional normothermia (figs. 9A and B). Mild and deep whole-body hypothermia were more effective in suppressing GFAP levels in CCI rats than mild and deep regional hypothermia, respectively (fig. 8).

Fig. 8. Western blot analysis showing the levels of glial fibrillary acidic protein (GFAP) in the ipsilateral cuneate nucleus (CN) on day 7 after chronic constriction injury (CCI) in rats treated with regional or whole-body hypothermia. (A) Application of regional hypothermia led to a significant decrease in GFAP levels (P < 0.05, by two-way ANOVA). In rats pretreated with mild or deep regional hypothermia, there was a significant decrease in GFAP levels compared with those pretreated with regional normothermia (*P < 0.05, by Tukey test). In the 5 h postinjury group, GFAP levels in the CN were also significantly decreased in CCI rats treated with mild or deep regional hypothermia compared with those treated with regional normothermia (*P < 0.05, by Tukey test). Furthermore, when administered preinjury and 5 h postinjury, deep regional hypothermia was more effective in suppressing GFAP levels than mild regional hypothermia (*P < 0.05, by Tukey test). However, in 1 day, 3 days, and 5 days postinjury groups, no significant differences in the levels of GFAP in the CN were observed among rats that received regional normothermia, mild regional hypothermia, or deep regional hypothermia (P > 0.05, by Tukey test). (B) A significant decrease in GFAP levels was noted after applying whole-body hypothermia (*P < 0.05, by two-way ANOVA). In preinjury, 5 h, 1 day and 3 days postinjury groups, mild and deep whole-body hypothermia were effective in decreasing GFAP levels in the CN of CCI rats compared with whole-body normothermia (*P < 0.05, by Tukey test). On day 5 postinjury, deep whole-body hypothermia, but not mild whole-body hypothermia, resulted in a significant decrease in the levels of GFAP compared with whole-body normothermia (*P < 0.05, by Tukey test). Whole-body hypothermia, either mild or deep, resulted in lower GFAP levels than the corresponding regional hypothermia (P < 0.05, by two-way ANOVA). Regional and whole-body normothermia contributed to a similar increase in GFAP levels (P > 0.05, by two-way ANOVA). β-actin was used as a loading control. Error bars represent mean ± SD; n = 5 rats per treatment.
and B). Similarly, in the 5 h postinjury group, there was a significant decrease in the levels of tumor necrosis factor (TNF-α) (A) and interleukin (IL-1β) (B) observed after applying regional hypothermia (P < 0.05, by two-way ANOVA). In rats pretreated with mild or deep regional hypothermia, there was a significant decrease in TNF-α (A) and IL-1β (B) levels compared with those pretreated with regional normothermia (*P < 0.05, by Tukey test). Similarly, in the 5 h postinjury group, TNF-α (A) and IL-1β (B) levels in the CN were significantly decreased in CCI rats that received mild or deep regional hypothermia compared with those that received regional normothermia (*P < 0.05, by Tukey test). In addition, deep regional hypothermia administered preinjury and 5 h postinjury more effectively suppressed TNF-α (A) and IL-1β (B) levels than did mild regional hypothermia (P < 0.05, by Tukey test). However, in 1 day, 3 days, and 5 days postinjury groups, the levels of TNF-α (A) and IL-1β (B) in the CN did not differ significantly among rats that received regional normothermia, mild regional hypothermia, or deep regional hypothermia (P > 0.05, by Tukey test). Levels of TNF-α (C) and IL-1β (D) decreased significantly in response to application of whole-body hypothermia (P < 0.05, by two-way ANOVA). Compared with whole-body normothermia, mild and deep whole-body hypothermia was effective in decreasing TNF-α (C) and IL-1β (D) levels in the CN of CCI rats in preinjury, 5 h, 1 day, and 3 days postinjury groups (P < 0.05, by Tukey test). In the 5 days postinjury group, a significant decrease in levels of TNF-α (C) and IL-1β (D) in CCI rats receiving deep whole-body hypothermia, but not mild whole-body hypothermia, compared with those receiving whole-body normothermia (P < 0.05, by Tukey test). Lower levels of TNF-α (C) and IL-1β (D) were observed in rats treated with deep whole-body hypothermia than in those treated with mild whole-body hypothermia (P < 0.05, by Tukey test). Mild and deep whole-body hypothermia more effectively decreased TNF-α and IL-1β levels than mild and deep regional hypothermia, respectively (P < 0.05, by two-way ANOVA). Regional and whole-body normothermia had similar effects on levels of TNF-α (C) and IL-1β (D) (P > 0.05, by two-way ANOVA). Error bars represent mean ± SD; n = 5 rats per treatment.
thermia (figs. 9C and D). In the preinjury, 5 h, 1 day, and 3 days postinjury groups, TNF-α and IL-1β levels were significantly decreased in the ipsilateral CN of CCI rats treated with mild or deep whole-body hypothermia compared with those treated with whole-body normothermia (figs. 9C and D). In the 5 days postinjury group, the levels of TNF-α and IL-1β showed a significant decrease in CCI rats receiving deep whole-body hypothermia, but not mild whole-body hypothermia, compared with those receiving whole-body normothermia (figs. 9C and D). Moreover, the levels of TNF-α and IL-1β were significantly lower in CCI rats that received deep whole-body hypothermia than in those that received mild whole-body hypothermia (figs. 9C and D).

TNF-α and IL-1β levels decreased more profoundly in CCI rats receiving deep whole-body hypothermia than in those receiving deep regional hypothermia (fig. 9). A similar finding was also observed when CCI rats were applied with mild regional and whole-body hypothermia.

**Discussion**

This study demonstrates that various degrees of regional hypothermia administered preinjury or 5 h postinjury attenuated the development of neuropathic pain behaviors and glial activation responses in the CN following median nerve CCI; however, regional hypothermia applied 1, 3, and 5 days postinjury failed to suppress neuropathic pain and glial activation. Further, various degrees of whole-body hypothermia could ameliorate nerve injury-induced neuropathic pain and decrease glial activation in the CN when applied in a wider time frame, from preinjury to day 3 postinjury. On day 5 postinjury, only deep whole-body hypothermia was capable of modulating neuropathic pain and glial activation. Deep hypothermia, either regional or whole-body, was more effective in attenuating neuropathic pain and glial activation than mild hypothermia.

Glial activation in the central nervous system is a common phenomenon following peripheral nerve injury that may contribute to the development of neuropathic pain. The activation of microglia and astrocytes is accompanied by up-regulation of the surface antigen, complement receptor 3, and the intermediate filament protein, GFAP, respectively. We assessed the glial activation response in the CN following median nerve CCI using immunocytochemical detection of OX-42 monoclonal antibody, which recognizes complement receptor 3, and GFAP monoclonal antibody. Previous studies showed that the activation of microglia and astrocytes reaches a peak and that mechanical hypersensitivity is most pronounced on day 7 after median nerve injury. Therefore, we chose day 7 as the time-point for experimental observations in the present study.

During the past decade, several clinical studies have provided evidence that systemic or focal hypothermia could confer protection against the pathologic consequences of tissue damage after central nervous system injury. However, the protective effects of hypothermia on peripheral nerve injury in humans or animal models have not been reported. Several studies have demonstrated that hypothermic treatment can inhibit the elevation of excitatory amino acids in ischemic regions of the brain, as well as microglial activation in the gray matter of the spinal cord after spinal cord compression injury and posttraumatic inflammatory cascades in brain tissue, such as IL-1β, inducible nitric oxide synthase, and superoxide. In the present study, preinjury treatment with regional or whole-body hypothermia decreased glial activation in the CN and ameliorated nerve injury-induced neuropathic pain. Furthermore, regional or whole-body hypothermic treatment before median nerve injury attenuated the discharge activity of the injured nerve. Electrophysiological studies have shown enhanced expression of persistently activated sodium channels in nerve fibers following nerve injury, which subsequently contributes to the generation of ectopic discharges and the release of glutamate. Pharmacological studies have demonstrated that the activated phenotype of cultured glia is observed after exposure to excitatory amino acids, such as glutamate and aspartate. Based on these observations, it is thought that using preinjury hypothermia to block injury-induced ectopic discharges in the median nerve would reduce glutamate release and thus inhibit the activation of glia and the development of neuropathic pain.

Mitsui et al. reported that early postischemic treatment (ischemia for 1 h), but not late postischemic treatment (ischemia for 3 h), with mild or deep regional hypothermia reduced fiber degeneration and endoneurial edema in a model of ischemic neuropathy. This is consistent with the findings of Kawamura et al. that inflammatory responses after peripheral nerve ischemia were attenuated in rats given mild or deep regional hypothermia immediately or 1 h postischemia, but not 3 h postischemia. The present study showed that mild or deep regional hypothermia administered 5 h postinjury could reduce glial activation and suppress neuropathic pain following CCI of the median nerve; however, regional hypothermia applied 1, 3, and 5 days postinjury had no such effects. In previous studies, we examined the glial activation responses in the CN after median nerve CCI at different time-points, and found that the levels of OX-42 and GFAP evidently increased as early as 1 day and 3 days after nerve injury, respectively. When regional hypothermia was administered 1, 3, and 5 days after CCI, microglia in the CN had already been activated. Conversely, microglia were still inactive when regional hypothermia was applied 5 h after CCI. It was speculated that regional hypothermia could inhibit microglial activation, but could confer no effects on already-activated microglia, even though postinjury administration of regional hypothermia remained effective in suppressing the generation of ectopic discharges following nerve injury in the electrophysiological studies. Further, high levels of proinflammatory cytokines, such as TNF-α and IL-1β, were detected in the CN of CCI rats treated with regional hypothermia 1, 3, and 5 days, but not 5 h, postinjury. There
is evidence that activated microglia produce cytotoxic molecules, including reactive oxygen species, nitric oxide, prostaglandins, and a variety of proinflammatory cytokines.\textsuperscript{41} An electrophysiological study of superficial dorsal horn neurons showed that TNF-\(\alpha\) enhances excitatory synaptic transmission by increasing the frequency of spontaneous excitatory postsynaptic currents, and IL-1\(\beta\) can both enhance excitatory synaptic transmission and reduce inhibitory synaptic transmission.\textsuperscript{29,42} The results suggest a pivotal role of proinflammatory cytokines in modulating synaptic plasticity and neuronal excitability, which subsequently cause central sensitization and contribute to the development of neuropathic pain. In addition, Gao and Ji\textsuperscript{29} reported that in response to nerve injury, microglia produced TNF-\(\alpha\), which activated astrocytes via c-Jun N-terminal kinase. Therefore, regional hypothermia may not have an effect on already-activated microglia; consequently, the release of proinflammatory cytokines by activated microglia contributes to the activation of astrocytes and the development of neuropathic pain.

Several studies have shown that whole-body hypothermia could prevent secondary neuronal damage, which was initiated through proinflammatory cytokine release from activated microglia following traumatic brain injury.\textsuperscript{43,44} Schmitt et al.\textsuperscript{45} reported that hypothermic treatment diminished release of TNF-\(\alpha\) and nitric oxide from activated microglial cells in an \emph{in vitro} cell culture model. The present study showed that whole-body hypothermia applied after nerve injury was effective in attenuating neuropathic pain behaviors and glial activation responses in the CN. The exact mechanism underlying these phenomena remains uncertain; however, it is thought that nerve injury initiates a signaling cascade in the nervous system and whole-body hypothermia plays a significant role in several aspects of the cascade. At an early stage, or before ectopic discharge-triggered microglial activation, whole-body hypothermia could suppress injury-induced ectopic discharges and consequently inhibit the activation of glia and the development of neuropathic pain. At a later stage, or after the initiation of microglial activation, whole-body hypothermia could inhibit proinflammatory cytokine release from activated microglia and remain effective in diminishing neuropathic pain and glial activation. However, a diminishing effect of whole-body hypothermia on neuropathic pain modulation and glial activation was observed when hypothermic treatment was given at an even later time. We speculate that the interactions between neurons, glia, cytokines, and central pain processing become increasingly complex and widespread during this time, and whole-body hypothermia alone is no longer able to tackle all these mechanisms of neuropathic pain.

In clinical applications investigating the therapeutic effects of regional hypothermia on extremity injury or musculoskeletal trauma, limb temperature is usually reduced to 28–32°C or even lower.\textsuperscript{46–48} However, only mild hypothermia, either alone\textsuperscript{49} or in combination with a potential neuroprotective agent,\textsuperscript{49} has been used for whole-body therapeutic treatment of central nervous system injury in real patients because lower temperatures could result in potentially life-threatening complications, including infection, arrhythmia, hypokalemia, coagulopathy, and heart failure.\textsuperscript{50} To investigate its therapeutic effects in animal models, whole-body hypothermia has been induced to as low as 21°C.\textsuperscript{31,52} Therefore, it is speculated that the adaptation to and tolerance of low body temperature varies among different species. Our study revealed that after nerve injury, whole-body hypothermia was more effective in attenuating neuropathic pain and glial activation than regional hypothermia. Further, deep whole-body hypothermia resulted in better therapeutic effects on pain than mild whole-body hypothermia. Based on these observations, a combination approach that includes mild therapeutic hypothermia and neuroprotective medications, which exploits the advantages and avoids the disadvantages of lowering body temperature, is suggested for the treatment of peripheral nerve injury. Thus, it is important to choose an appropriate neuroprotectant for a given time-point in patients with peripheral nerve injury because the results of the current study demonstrate that a variety of cells and mediators play pivotal roles in neuropathic pain development during different phases of nerve injury.

In conclusion, the present study demonstrates that in the early stage after nerve injury, ectopic discharges from injured nerves play an important role in pathologic pain states, and the suppression of ectopic discharges effectively attenuates glial activation and neuropathic pain. Once microglia are activated, a variety of cytokines are released, which subsequently contribute to paracrine activation of the surrounding glial cells and the development of neuropathic pain. Hence, it is mandatory to modulate the cytokines involved in subsequent pain processing to achieve optimum pain relief. This study offers evidence to corroborate the concept and importance of choosing an appropriate therapeutic modality based on the stage of nerve injury when applying therapeutic hypothermia for neuropathic pain.

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Analgine by H. K. Mulford of Philadelphia

Promoting themselves as “Factors of Reliable Tablets” in their advertisements, the Philadelphia pharmaceutical firm of H. K. Mulford compounded pain-relieving Analgine. Stacked in corked cylinders of glass (above), these five-grain compressed tablets were targeted at relieving migraine, neuralgia, and rheumatic pains. Marketed by 1896 as “a most reliable analgesic” that would “not depress the heart,” Analgine became surprisingly popular with the public. Perhaps people enjoyed the proprietary blending of “Acetanilid” with a salicylate and caffeine. Or maybe some customers appreciated the extract of hyoscyamus. Or did the quarter-grain of cannabis per tablet have something to do with Analgine’s success? (Copyright © the American Society of Anesthesiologists, Inc. This image also appears in the Anesthesiology Reflections online collection available at www.anesthesiology.org.)

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