

Development of Neuropathic Pain in the Rat Spared Nerve Injury Model Is Not Prevented by a Peripheral Nerve Block

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Background: The mechanisms responsible for initiation of persistent neuropathic pain after peripheral nerve injury are unclear. One hypothesis is that injury discharge and early ectopic discharges in injured nerves produce activity-dependent irreversible changes in the central nervous system. The aim of this study was to determine whether blockade of peripheral discharge by blocking nerve conduction before and 1 week after nerve injury could prevent the development and persistence of neuropathic pain-like behavior in the spared nerve injury model.

Methods: Bupivacaine-loaded biodegradable microspheres embedded in fibrin glue were placed in a silicone tube around the sciatic nerve to produce a conduction block. After sensory-motor testing of block efficacy, a spared nerve injury procedure was performed. Development of neuropathic pain behavior was assessed for 4 weeks by withdrawal responses to stimulation (*i.e.*, von Frey filaments, acetone, pinprick, radiant heat) in bupivacaine microspheres-treated animals ($n = 12$) and in controls ($n = 11$).

Results: Bupivacaine microspheres treatment produced conduction blockade with a complete lack of sensory responsiveness in the sural territory for 6 to 10 days. Once the block wore off, the degree of hypersensitivity to stimuli was similar in both groups.

Conclusions: Peripheral long-term nerve blockade has no detectable effect on the development of allodynia or hyperalgesia in the spared nerve injury model. It is unlikely that injury discharge at the time of nerve damage or the early onset of ectopic discharges arising from the injury site contributes significantly to the persistence of stimulus-evoked neuropathic pain in this model.

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PERIPHERAL nerve injury may induce severe and intractable pain. Attempts have been made to prevent the development of persistent pain in patients undergoing limb amputation or thoracotomy.¹⁻³ Although anecdotal reports suggest that phantom pain can be prevented with a neuraxial nerve block, these trials remain controversial.⁴⁻⁷

When a nerve is transected, a high-frequency discharge is elicited from the damaged axons.⁸⁻¹⁰ This lasts for several minutes, and it has been argued that this may be sufficient to induce excitotoxic damage in central neurons by virtue of the excessive release of excitatory transmitters, particularly glutamate.¹¹ Glutamate acts on ligand-gated AMPA (α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and NMDA (*N*-methyl-D-aspartate) ion channel receptors as well as on metabotropic receptors. Calcium entry *via* the NMDA channels is thought to play a major role in excitotoxicity by overwhelming intracellular buffering capacity. The effect of acute injury discharge may be augmented by ectopic activity generated after a nerve lesion in injured and neighboring uninjured neurons.¹² The potential source of such ectopic input is diverse, including the site of injury (neuroma), along the nerve, and in the dorsal root ganglia.¹³⁻¹⁵ The abnormal afferent inflow generated at the time of peripheral nerve injury may result in neuropathic pain.

The time course of neuropathic symptoms varies in different animal models of neuropathic pain,¹⁶ as does the level of ectopic discharges. After a complete sciatic nerve transection, ectopic activity in A β and A δ fibers is prominent during the first 2 weeks, followed by C-fiber ectopic discharges.¹³ The possible effects of blocking the injury discharge on the development of neuropathic pain have been studied. The degree of autotomy (self-mutilation) is reduced after sciatic transection when the nerve is bathed in local anesthetic before being cut,¹⁷ and short-duration conduction block of the nerve before injury lessens the severity of heat hyperalgesia due to partial constriction of the sciatic nerve.¹⁶ In other rat models, such as partial transection of the sciatic nerve¹⁸ or spinal nerve ligation,¹⁹ however, a single dose of local anesthetic failed to prevent long-term neuropathic pain.^{16,20}

To block injury discharge and the spontaneous discharge originating from the injury site, a long duration of peripheral nerve blockade is required.²¹ To achieve this, we used biodegradable polymeric microspheres loaded with bupivacaine. The microspheres permit a long-lasting continuous drug delivery for more than 1 week *in*

vitro and a long duration of nerve blockade *in vivo* while the polymer slowly degrades.²²

The aim of the current study was to identify whether the injury discharge and peripheral ectopic activity arising from the injury site in the first days after the injury contribute to the development of pain hyperexcitability. A proximal action potential block was established before performing spared nerve injury (SNI) surgery,²³ and the development of neuropathic pain-related behavior was observed for 4 weeks after creation of the lesion.

Materials and Methods

The experiments were approved by the Committee on Animal Experimentation of the Canton of Vaud, Lausanne, Switzerland, in accordance with Swiss Federal law on animal care and the guidelines of the International Association for the Study of Pain.²⁴ Adult male Sprague-Dawley rats (Charles River, L'Abresle, France) initially weighing 300 g were used for the experiments. They were housed four to five per cage with thick sawdust bedding, water and food *ad libitum*, and a 12-h day/night cycle.

Experiments

Effect of Bupivacaine-loaded Microspheres on the Development of Neuropathic Pain. Animals were randomly assigned to a bupivacaine-loaded microspheres-treated group (n = 12) or control group (n = 11). Once the block was documented, SNI surgery was performed.

Control Experiments. A preliminary development phase was required to prove that selective long-term blockade of sciatic nerve terminal branches was possible without a lesion to the nerves. Instead of percutaneous injection or direct application of the microspheres around the nerve, we embedded them in fibrin glue and poured them in a silicone tube previously placed around the intact sciatic nerve proximal to the trifurcation in sural, common peroneal, and tibial nerves. The microspheres were thus trapped in the tube. The duration of nerve blockade and the recovery of sensitivity were documented (n = 3). To investigate the impact of the procedure itself (*i.e.*, surgery, tube, fibrin glue) on basal pain sensitivity, behavioral tests were conducted in naive (n = 4) and SNI rats (n = 4) after implantation of the silicone tubes filled with fibrin glue (sham procedure), and the results were compared with the test results obtained in their respective control animals (n = 4 in each group). Macroscopic appearance and histologic nerve preparations were examined.

Surgery

Nerve Block. Under 1.5–3.0% isoflurane (Forene; Abbott, Baar, Switzerland) anesthesia, the skin was incised

from the left greater trochanter to the knee. The muscle layers were separated between the gluteus superficialis and the biceps femoralis, exposing the sciatic nerve from the emergence of the musculocutaneous branch to the trifurcation in sural, tibial, and peroneus branches. A silicone tube (11 mm long) was incised on its long axis, kept open by two forceps, and placed carefully below the sciatic nerve. From the elastic force of the silicone material, the tube closed around the nerve. Two 5-0 silk ligatures were wound around the tube to close the longitudinal slit, which was then sealed with fibrin glue (Tissucol; Baxter, Volketswil, Switzerland). A fibrin glue plug was placed at the distal end. A 50- μ l fibrin solution containing the bupivacaine microspheres was then slowly poured inside the tube through the opposite open end. Special care was taken to avoid any stretch or damage to the sciatic nerve and its branches. Muscle and skin were closed in two layers, and the animals were allowed to recover. The control group had the same surgery except that no bupivacaine-containing microspheres were inserted into the silicone tube. All the animals received a preventive subcutaneous injection of 150 mg/kg amoxicillin.

Spared Nerve Injury. The skin and muscles were reopened using the same wound. The common peroneal and tibial nerves were exposed and ligated with a 5-0 silk suture; 2–3 mm of the nerves was removed below the ligations, with special care taken to avoid any damage to the sural nerve.²³

Bupivacaine-loaded Microspheres

The microspheres were of the following composition: 75% wt/wt bupivacaine, 24.95% wt/wt polylactic–glycolide polymer, and 0.05% wt/wt dexamethasone. Microspheres were formulated in a commercial facility as described previously²⁵; bench-top formulations with almost identical *in vivo* activity can be formulated as described previously.^{22,26} The fibrin glue²⁷ used (Tissucol) consisted of two separate components, fibrin and thrombin, that form a fibrin seal once combined. The microspheres were diluted in the fibrin compound of fibrin glue at a final concentration of 300 mg/ml. The silicone tube was filled with 50 μ l of this solution (treated group) or fibrin alone (sham procedure, control group).

Motor and Sensory Conduction Block Testing

The rats were gently held with a cloth wrapped above their waist to restrain upper extremities, and various sensitivity measurements were performed on both hind limbs.

Noxious Stimulation. The skin on both sides of the hind paws (sural and saphenous nerve territories) was pinched with a small forceps, and the withdrawal reflex response (yes or no) was noted.²⁸ The rats were then positioned to stand with one hind paw on a 56°C hot plate while the other was held by the experimenter.

Table 1. Effect of Sham Procedure on Naive and Neuropathic Animals

	Naive	Sham Procedure	SNI	SNI + Sham Procedure
Mechanical allodynia threshold (g)	11.35 ± 2.51	12.40 ± 15.00	0.02 ± 0.01	0.03 ± 0.01
Mechanical hyperalgesia duration (s)	0.50 ± 0.00	0.50 ± 0.00	16.96 ± 15.35	10.4 ± 3.04
Cold allodynia duration (s)	0.38 ± 0.25	0.38 ± 0.25	43.05 ± 25.71	48.02 ± 23.96

Control experiments: Pain sensitivity assessment 1 week after the different procedures in naive and SNI animals and their respective sham-operated controls to prove the harmlessness of the block surgery technique without bupivacaine microspheres. No difference due to the sham procedure was observed in naive or SNI rats (n = 4 in each group).

SNI = spared nerve injury.

The time required to withdraw the hind paw from the hot plate was recorded. A cutoff at 12 s was chosen to avoid injury.²⁹

Proprioception and Motor Function. Three additional tests were used to ensure that proprioceptive and motor functions are blocked. Briefly, hopping response is the ability of the animals, while standing on one leg with their body being moved laterally, to hop in the direction of the movement (score: 0 = no hopping, 1 = successful hopping). The tactile placing response shows the capability to reposition the paw after extension (1–4 score range: 1 is complete repositioning, 4 is no repositioning, and 2 and 3 are intermediate positions). Extensor postural thrust measures the weight an animal can place on its hind limb (score: 1 = more than 100 g, 2 = 50–100 g, 3 = 20–50 g, 4 = less than 20 g).³⁰ All tests were performed once daily during the period of the block.

Pain Sensitivity Assessment

Baseline behavioral testing was made after a 1-week period of habituation to the environment and observer.²³ Both right (noninjured) and left (injured) hind paws were tested for each modality. To avoid the effect of the circadian cycle, all behavioral assessments were performed during the same period (7:30–11:00 AM). The investigator was not aware of the treatment applied. During the period of nerve block, the observer would, however, be aware of the treatment because of the motor paralysis. To ensure blinding in the postblock phase, new numbers were assigned to the animals once the block wore off. Treated and control animals were tested during the same session. After SNI surgery, mechanical and cold sensitivity were recorded twice a week for 4 weeks. Plantar heat stimulation was restricted to days 18 and 26 so as to avoid heat-induced injury.

Mechanical Allodynia. Light mechanical stimuli were applied to the foot using a series of von Frey monofilaments in an ascending order.²³ Testing began using the lowest force filament (0.0174 g). The filament was pressed perpendicularly against the skin until it bent. The filament was applied five times every 3 s, and if there was no withdrawal response, the next higher stimulus was tried. The threshold (in grams) was defined as the lowest force that evoked a brisk withdrawal response to at least one of five stimuli.

Mechanical Hyperalgesia. The pinprick test was performed on the lateral part of the plantar surface using a safety pin. A single prick was given at a force such that the skin dimpled but was not penetrated. The duration of paw withdrawal was recorded with a minimal time at 0.5 s for brief response and a maximal cutoff at 60 s.

Cold Allodynia. A drop of acetone was gently applied on the lateral side of the foot without touching the skin with the syringe, and the cumulative duration of paw withdrawal after the cold sensation experienced after evaporation of acetone was measured over a period of 120 s after the initial stimulus.

Radiant Heat Test. Rats were placed in bottomless, clear, plexiglas, rectangular chambers on a glass shelf. A movable radiant heat source enabled us to stimulate the lateral part of the hind paw. We measured the duration of withdrawal due to the heat stimulation with a minimal time of 0.5 s and a maximal cutoff at 60 s.

Data Analysis and Statistics

The behavioral data were expressed as mean ± SD (range) of the recorded threshold (grams) or duration (seconds) withdrawals. A two-way analysis of variance, with time treated as a repeated-measures factor, was made to analyze the difference between the groups after the block. $P < 0.05$ was declared significant. A *post hoc t* test with Bonferroni correction was used when appropriate (JMP software, version 3.1.5; SAS Institute, Cary, NC).

Results

Control Experiments

The effect of bupivacaine microspheres along the intact nerve was measured by motor and sensory testing. Once the block wore off (after 8.4 ± 1.1 days), all animals recovered their basal level of motor and sensory responsiveness. After the sham procedure on intact sciatic and SNI-injured nerves, pain sensitivity assessed by von Frey hair, pinprick, acetone, and heat was not modified (n = 4 in each treated and control group) (table 1). Macroscopic examination of the sciatic nerve revealed no signs of nerve damage or constriction. Microscopic examination showed no signs of pathologic demyelination, edema, or invasion of inflammatory cells within the nerve (data not shown).

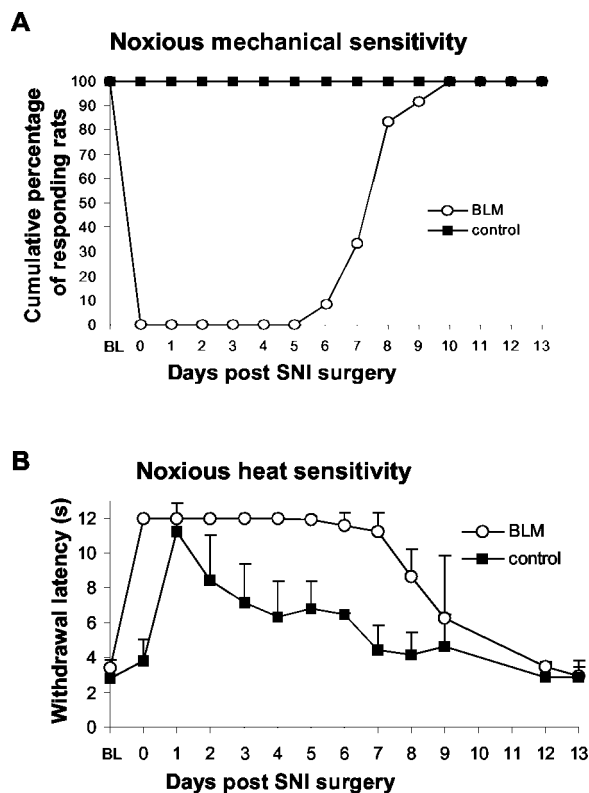


Fig. 1. Nerve block evaluation: response to noxious pinch mechanical stimulation in the sural nerve territory (A) and to noxious 56°C thermal stimulation (B) for the bupivacaine-loaded microspheres (BLM)-treated group (n = 12) and the control group (n = 11). (A) The control animals all displayed a normal response, whereas bupivacaine-loaded microspheres-treated animals all showed no response until day 5. From days 6 to 10, the animals in the treated group gradually recovered from the nerve block. (B) Until day 5, all animals in the bupivacaine microspheres group reached the cutoff of 12 s. Then, progressively, the withdrawal duration of the treated animals approached the withdrawal duration of control animals. Notice changes in latency for the control animals due to motor dysfunction and inability to bear weight properly on the hot plate induced by spared nerve injury (SNI) surgery. The control animals never reached the cutoff time.

Effect of Bupivacaine Microspheres-induced Block in the Spared Nerve Injury Model

Animals showed normal exploratory activity and responsiveness. On completion of the study, the surgical site was checked macroscopically for nerve injury or infection. All the tubes were in place, without indication of damage, constriction, or compression.

Nerve Block. Before the SNI surgery (at t = 0, 3 h after insertion of the bupivacaine microspheres), all animals in the bupivacaine microspheres group (n = 12) demonstrated complete sensory-motor nerve blockade. The sciatic nerve territory was unresponsive to mechanical and thermal nociceptive stimulation. Proprioception and motor function were also abolished, with scores of 0 for the hop, 4 for the flip, and 4 for the weight-bearing tests. In both groups, the SNI procedure induced sensory-motor alterations due to the injury to the tibial and common peroneal nerves and the hamstring muscles.

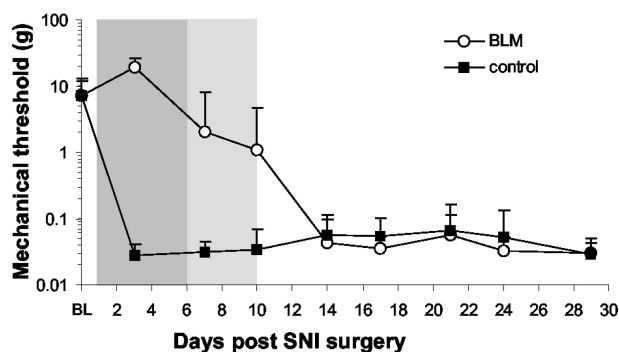


Fig. 2. Response to mechanical stimulation with von Frey filaments in the sural skin territory for bupivacaine-loaded microspheres (BLM)-treated group (n = 12) and spared nerve injury (SNI) control animals (n = 11). The control animals demonstrated a marked decrease in mechanical withdrawal threshold after SNI surgery ($P < 0.01$ compared with baseline). The treated group showed an increase in threshold (corresponding to the weight of the paw passively lifted by the von Frey filament) while all animals were blocked (dark gray zone), a mean intermediate response when some animals lost their block (light gray zone), and marked hypersensitivity when all blocks faded ($P < 0.001$ compared with baseline). No statistically significant difference was observed between the control and bupivacaine-treated groups after day 10 ($P > 0.05$).

This impaired the flip, hop, and weight-bearing tests (data not shown). Signs of a nerve conduction block of the left sciatic territory were nevertheless evident by absence of mechanical and thermal nociceptive-induced reflexes for 7.9 ± 0.2 days (6–10 days) in the bupivacaine microspheres group (fig. 1). The sensitivity of the saphenous territory to pinch stimulation was not changed.

Mechanical Allodynia. The withdrawal threshold to non-nociceptive mechanical stimulation of the sural skin territory dropped substantially in all control animals after SNI surgery (fig. 2). During the first phase of nerve blockade (fig. 2, dark gray zone), the withdrawal threshold of the bupivacaine microspheres-treated animals increased. During the second phase, as the nerve

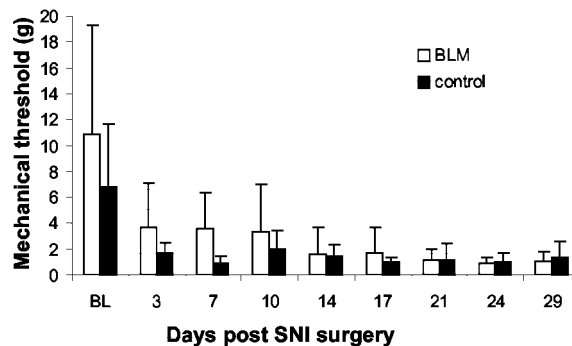


Fig. 3. Response to mechanical stimulation with von Frey hair in the saphenous skin territory for bupivacaine-loaded microspheres (BLM)-treated (n = 12) and spared nerve injury (SNI) control animals (n = 11). Both groups display a rapid decrease in threshold ($P < 0.05$ between baseline and each group at day 14) without a significant difference between the groups ($P > 0.05$) after day 14.

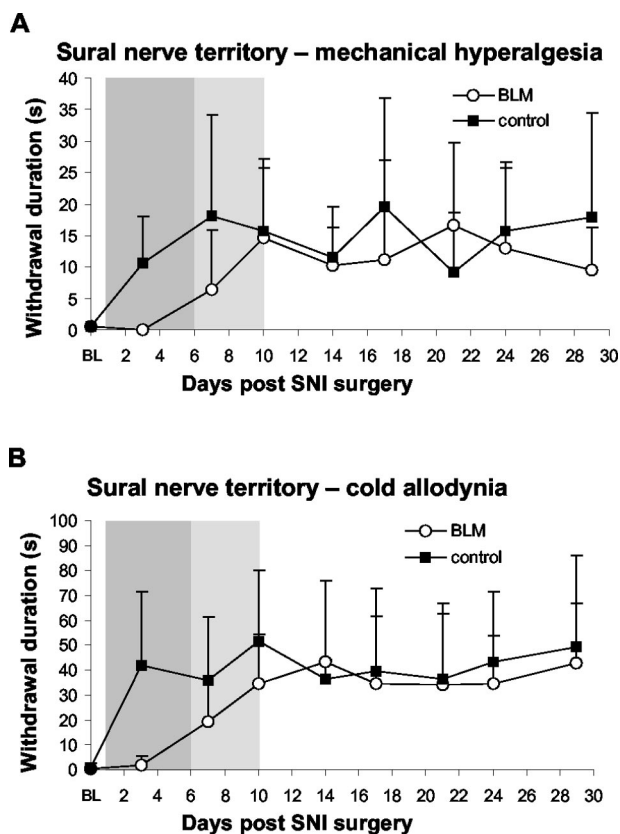


Fig. 4. Paw withdrawal duration after pinprick and cold stimulation. (A) The control group ($n = 11$) developed mechanical hyperalgesia with an increased withdrawal duration compared with baseline ($P < 0.001$). Bupivacaine-loaded microspheres (BLM)-treated animals ($n = 12$) showed no response during the nerve block phase (dark gray zone) and intermediate response when some animals were already unblocked (light gray zone). After 10 days, they expressed the same degree of hyperalgesia as the control group ($P > 0.05$ comparing control *vs.* treated animals, $P < 0.05$ compared with baseline). (B) The control group developed marked allodynia to innocuous cold stimulation compared with baseline recordings ($P < 0.001$). The BLM-treated animals showed no response while all animals were blocked (dark gray zone) but then increased their withdrawal duration when some animals lost their block (light gray zone) and demonstrated the same degree of allodynia ($P < 0.005$ compared with baseline) after day 10, with no significant difference from the control group ($P > 0.05$). SNI = spared nerve injury.

block faded, the mean withdrawal threshold decreased. By day 10 (third phase, *white zone*), when all of the bupivacaine microspheres-treated animals had recovered from the nerve blockade, the withdrawal threshold dropped, and no difference was observed between bupivacaine microspheres-treated and control animals ($P > 0.05$).

The saphenous nerve is a femoral nerve branch; therefore, sensitivity in its skin territory was not impaired by the sciatic nerve blockade. Control and bupivacaine microspheres-treated groups showed a similar increase in sensitivity to mechanical stimulation after SNI once the block wore off (days 14–29, $P > 0.05$) (fig. 3). One statistically significant difference ($P = 0.025$) was ob-

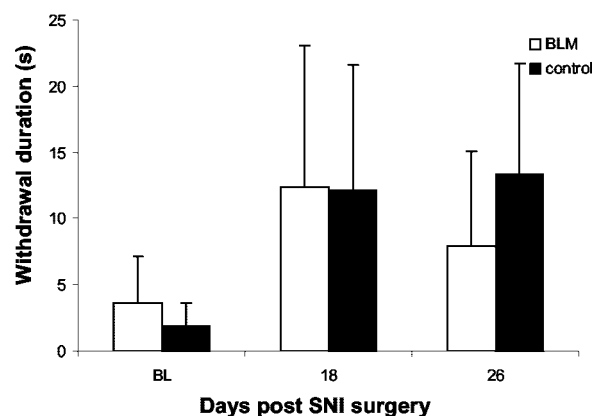


Fig. 5. Paw withdrawal duration after radiant heat stimulation in the sural nerve territory. Both groups (control and bupivacaine-loaded microspheres [BLM]-treated) developed hyperalgesia after the nerve injury ($P < 0.05$ compared with baseline) without a statistically significant difference between the groups ($P > 0.05$). SNI = spared nerve injury.

served between the two groups during the period of nerve blockade at one time point (day 7). Because the observer was not blinded to the treatment during the block, the biologic significance of this finding cannot be assessed.

Mechanical Pinprick Hyperalgesia. After the SNI procedure, control animals rapidly developed mechanical hyperalgesia (fig. 4A). In the bupivacaine microspheres-treated group, during the nerve blockade, a complete lack of responsiveness was present at day 3 and only a partial response was elicited at day 7. Beyond 7 days, however, the withdrawal response to a pinprick was similar to that in the control group ($P > 0.05$).

Cold Allodynia. The duration of withdrawal after the cold stimulation increased significantly in the control group at day 3. The bupivacaine microspheres-treated animals showed no response during the block, but after it was over, a cold allodynia-like behavior identical to that in the control group was observed ($P > 0.05$) (fig. 4B).

Heat Hyperalgesia. All control animals developed heat hyperalgesia. After recovery from the nerve conduction block, no statistically significant difference in the withdrawal duration was recorded between bupivacaine microspheres-treated and control groups ($P > 0.05$) (fig. 5).

Discussion

When applied directly to the sciatic nerve, bupivacaine-loaded microspheres produced a peripheral conduction blockade that lasted for 6 to 10 days. This is much longer than a single direct application of bupivacaine, in which the block only lasts for several hours.²⁸ Although delivery of local anesthetics by a constant infusion to the nerve can produce a long blockade, the dose administered is high and there is a chance of sys-

temic effects. Our technique was used to produce a dense local sciatic nerve conduction block before and for several days after SNI surgery. The treatment was likely to block both the immediate injury discharge produced at the time of injury and any ectopic activity arising from the injury site (acute neuroma) in the periphery for 6–10 days. The major finding of the study is that the intensity of the SNI-induced pain hypersensitivity in the sural nerve territory was effectively identical in the experimental and control animals as soon as the block wore off.

The amount of discharges in each of the different fibers after a peripheral nerve axotomy varies with time.^{13,31} C-fiber action potentials were recorded during 15 min after nerve transection,⁹ followed within hours to days by a predominant spontaneous firing and mechanical excitability of myelinated afferents.^{32–34} In the spinal nerve ligation model, uninjured and injured afferents participate in spontaneous activity.^{12,35} Our results suggest that peripheral activity at the time of injury and arising from the injury site during the first week is not a major contributor to persistent neuropathic pain in this model. Early ectopic activity proximal to the block arising within the dorsal root ganglion^{14,36} may contribute to the development of pain hypersensitivity, or delayed peripheral ectopic activity once the block wears off (present after 1 week) may induce pain hypersensitivity.

Different neuropathic models may have a differential sensitivity to “preemptive nerve block.” Short-duration peripheral nerve block produced by acute local anesthetic application delayed the onset and decreased the magnitude of autotomy in total denervation models (sciatic axotomy or sciatic plus saphenous axotomies).^{17,37} It also delayed the appearance of neuropathic behavior in the chronic constriction injury³⁸ model.^{16,39} Such a block had no effect on the long-term development of stimulus-evoked pain in the spinal nerve ligation¹⁹ or partial sciatic nerve ligation¹⁸ models, however.^{16,20,40} This seems to indicate that acute injury discharge has a minimal role in the establishment of stimulus-evoked neuropathic pain. The relevance of autotomy as an outcome signal for pain is controversial,⁴¹ but it might mean that spontaneous pain behavior responds differently than stimulus-evoked pain to acute nerve block at the time of injury.

Our study partially mimics clinical situations, in which the conduction nerve block is strong before and during the surgery, but during the postoperative period, the block is progressively decreased to avoid motor dysfunction. Some clinical trials that used peripheral nerve block to abort the development of phantom limb pain showed no effect.⁴² We suggest that such short-term peripheral blockade is unlikely to prevent the development of persistent stimulus-evoked pain after partial nerve injury, but it remains an efficient treatment for acute postoperative pain.

Acute injury and ectopic discharges arising from the neuroma are only one of several potential mechanisms that may be responsible for the development of neuropathic pain-related behavior. Excessive peripheral inputs have been proposed to induce the death of inhibitory cells in the dorsal horn.¹¹ Our data do not exclude the possibility that locations proximal to the block might produce sufficient ectopic discharge to induce such cell death or that cell death is not activity-dependent. Data show both a loss of inhibitory synaptic transmissions and activation of apoptosis in the dorsal horn after SNI and chronic constriction injury, but not after complete sciatic nerve transection.⁴³ Other mechanisms are possible, including nonactivity-dependent transcriptional changes in the dorsal root ganglion and dorsal horn neurons, dependence of the pain sensitivity on ongoing ectopic activity to drive central sensitization, neuron-glia interactions, and altered descending controls.^{44,45} None of these would be altered by nerve blockade at the time of and for several days after a partial nerve injury.

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