

Foot Hyperalgesia after Thoracic Burn Injury —Histochemical, Behavioral and Pharmacological Studies—

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Using behavioral, immunohistochemical and pharmacological studies, we report here that large thoracic burn injuries remotely induce hindpaw hyperalgesia during the healing stage.

During 2–3 weeks after thoracic burn injury when the skin was regenerating from the wound, we observed by formalin test that the number of flinching behaviors significantly increased and simultaneously we observed by von Frey test that rats developed mechano-hyperalgesia in the foot. In the dorsal horn of the lumbar spinal cord in burn injured rats, c-Fos expression was significantly augmented after plantar formalin injection. The expression of μ -opioid receptor in burn injured rats was significantly decreased compared with that in sham operated rats. The expression of substance P and CGRP in the lumbar dorsal horn was not different between burn

and sham operated animals. We also observed that intrathecal administration of glutamate receptor antagonists (MK801 and CNQX) but not cyclooxygenase-2 antagonist (NS-398) reversed the threshold of von Frey test on the foot up to the control level at 2 weeks after injury.

Collectively, we analyzed a new pain model showing foot hyperalgesia after thoracic burn injury and demonstrated that neurotransmission of glutamate was enhanced at the lumbar spinal cord level by immunocytochemistry and intrathecal administration of NMDA and non NMDA antagonists.

Although the precise mechanism of how remote hyperalgesia at the healing stage developed in this model remains to be confirmed, substances such as trophic factors released from the regenerating skin may cause systemic hyperalgesia including in the foot.

Key words: Mechano-hyperalgesia, von Frey test, c-Fos, MOR, NMDA

I. Introduction

Burn injury is usually accompanied by pain at the injured skin. Burn pain follows the physical destruction of tissues by flames, scalding, electricity, radiation or chemical agents. In first-degree burns, in which the skin is reddened but not moist or blistered, secondary hyperalgesia in the undamaged tissue surrounding the injury was also reported as

well as primary hyperalgesia in a human model [14, 16].

A second- or third degree burn injury lyses cells in the local tissue and liberates virtually several kinds of mediators such as bradykinin, prostaglandins, interleukins and monoamines which may activate peripheral nociceptors. The immediate pain at the time of initial injury is due to the activation of thermal nociceptors that will be destroyed along with other tissues as the temperature rises [20].

On the other hand, during the acute period after a large burn injury, stress-induced analgesia is observed. In animal models, both the plasma β -endorphin level and tail flick latency were reported to increase within 6 hr after scald injuries above 15% of the total body surface area (BSA) [15]. Pain and these analgesia have been mainly studied during the acute period of burn injury and in areas around the damaged tissue. However, little attention was paid to

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other dermatomic areas of the body except for the injured skin area.

In the present study we analyzed the pain status of rats using behavioral tests such as formalin test and von Frey hair test at the hindpaw for 4 weeks after thoracic burn injury. The formalin test is widely used in behavioral studies. Formalin injected subcutaneously in the rat hindpaw elicits a biphasic pattern of flinching composed of an early acute period (phase 1) followed after a short delay by a second, longer period of sustained behavioral activity (phase 2) [3]. Phase 1 is generally attributed to a direct effect on peripheral nociceptors, whereas phase 2 is related to the subsequent development of inflammation and spinal cord sensitization [17]. Von Frey test was used for quantifying the mechanical and thermal sensitivity of the foot. A brisk withdrawal response to mechanical or thermal stimuli to the hindpaw was measured using a plastic probe (von Frey filament) [13, 18]. As the burn injured area was far away from the foot and hyperalgesia in phase 2 was observed by the formalin test, we next aimed to examine the histochemical change in the lumbar spinal cord. We investigated the expressions of c-Fos and opioid receptor to clarify the role of excitatory and/or inhibitory neurotransmission at the dorsal horn of the spinal cord. We also investigated pharmacologically the involvement of glutamate or prostaglandins in the transmission of nociceptive information at the lumbar dorsal horn by intrathecal administration of NMDA, non-NMDA and cyclooxygenase-2 (COX-2) inhibitors on the foot hyperalgesia.

II. Materials and Methods

Animals

Male Sprague–Dawley rats (SLC Co., Shizuoka, Japan), weighing 175–225 g were used. The rats were maintained on a 12 hr light/12 hr dark cycle. The ambient temperature was kept at 22°C, and the rats had free access to standard laboratory food and tap water. All experimental procedures were approved by our institution's Animal Investigation Committee and adhered to the ethical guidelines of the International Association for the Study of Pain.

Rat thermal injury model

Making the burn model was conducted according to a previous study with minor modifications [23]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and had their ventral and dorsal areas shaved. The back and the flanks of the chest were immersed in hot water 75–85°C for 10 sec while the ventral chest was exposed for only 6 sec. This exposure produced a full-thickness third degree burn to the skin and caused no injury to deeper tissues. The burn area percentage of BSA was calculated as 25% from the equations of the model described previously [15]. While recovering from the anesthesia, the injured animals were warmed by heat lamps and fluid and were resuscitated with 15 ml crystalloid solution administered intraperitoneally. One percent silver sulfadiazine cream (Geben®, Tokyo

Tanabe Inc., Tokyo, Japan) was applied to the injured area. The control animals were treated in the same way as the trauma group, except that they were immersed in lukewarm water.

Behavioral assessments

Spontaneous motor activity of each rat was measured with an electric activity meter (AUTOMEX-II, Columbus Instruments International Corporation, Ohio, USA) for 10 min at 1, 2 and 3 weeks after injury. Thermal hyperalgesia, mechano-allodynia and hyperalgesia, and cold allodynia were examined at the plantar surface of the rat hind paws. Nociceptive pain behavioral tests except thermal hyperalgesia were performed before burn-treatment and daily thereafter. The test of thermal hyperalgesia was examined at 1, 2 and 3 weeks after injury. The method in each behavioral test is detailed below.

Thermal hyperalgesia

Each rat was administered a subcutaneous injection of 50 µl of 5% formalin into the plantar surface of the left hindpaw using a 27-gauge syringe needle. Each rat was then immediately placed in a Plexiglas box (30×30×30 cm) positioned over a mirror angled at 45° to allow an unobstructed view of the paws by the observer. Observations to determine nociceptive responses began after placing the rat into the box and continued for the following 60 min. We evaluated the number of flinching behaviors in the rat hindpaw.

Mechanical hyperalgesia and allodynia

Each animal was confined under a Plexiglas box (8×8×18 cm) on a raised wire mesh platform. Mechanical stimulation was applied with two different von Frey filaments, 4.13 for mechano-allodynia and 4.93 for mechano-hyperalgesia (bending force corresponds to 12 and 47 mN, respectively) onto the ventral surface of the hindpaws [18]. A withdrawal of the hindpaw immediately after the von Frey stimulation was recorded as a response. A single trial consisted of 10 applications of one von Frey filament within approximately 30 sec. Percent response frequency was calculated for each trial using the following formula: (number of withdrawals/10)×100=% response frequency. Three trials were carried out for each von Frey filament per time point and a mean % response frequency was calculated [13].

Cold allodynia

Each rat was placed under a Plexiglas box on a wire-mesh platform similar to the von Frey test. A bubble of acetone was applied to the bottom of each hindpaw with a flat-tipped 18-gauge syringe needle [18]. Direct contact between the needle tip and the hindpaw was avoided. A rating scale was used as follows: 0; no withdrawal response 1; simply raised 2; several hindpaw shakes 3; shaking and licking. Three trials were carried out for each and a percent maximum possible response is calculated using the following formula: (rated score/3)×100=% maximum possible response. Then mean % maximum possible response was calculated.

Immunocytochemistry

Under deep anesthesia rats were perfused transcardially with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 at 60 min after 5% formalin (50 μ l) injection to the left hindpaw. The spinal cord was removed and postfixed in the same fixative for 12 hr. Then sections at the L4 and L5 segment (20 μ m in thickness) were cut on a cryostat. Sections were incubated in rabbit anti-c-Fos serum at a dilution of 1:1000 (Oncogene Science, MA, USA) in 0.1 M phosphate-buffered saline (PBS) containing 0.5% Triton X-100 at 4°C for 3 days. They were incubated overnight in biotinylated anti-rabbit IgG (1:500, Vector Labs) at 4°C and in avidin-biotin complex (1:1000, Vector Labs) for 2 hr at room temperature. Sections were exposed to 3,3'-diaminobenzidine-4HCl (20 mg/100 ml, Sigma) in 0.1 M Tris buffer, pH 7.4, containing 0.01% H₂O₂.

Light microscopic cell counts of c-Fos positive neurons were obtained at $\times 200$ magnification. The number of c-Fos positive neurons was counted in the left dorsal horn in laminae I–VI of both the burn and sham groups. Thirty sections picked up alternatively from the spinal cord at L4–5 of each animal were analyzed.

Detection of μ -opioid receptor (MOR) expression was carried out using the same procedure as described above (primary antiserum, raised in rabbit, dilution, 1:5000, Diasorin, MN, USA). To quantify the MOR immunoreactivity in both groups, we employed a computer-assisted analyzing system. The MOR immunoreactivity was measured as the optical density per area (O.D./ μ m²) using the NIH image software in the area where MOR neurons were distributed [7]. The O.D. in lamina III–IV of the same section was used as the background and this was subtracted from the O.D. of the MOR immunoreactivity. Thirty sections selected alternately from the spinal cord at L4–5 of each animal were analyzed.

To examine the primary nociceptive afferents from the foot, immunocytochemistry of substance P or calcitonin gene-related peptide (CGRP) (both primary antiserum, raised in rabbit, dilution, 1:1000, Yanaihara lab, Shizuoka, Japan) was processed in a similar way to MOR immunocytochemistry.

Intrathecal administration

Intrathecal catheters were implanted in rats under isoflurane anesthesia according to a procedure previously described [6] with a slight modification. A polyethylene catheter (PE-10) was inserted through an incision in the atlanto-occipital membrane and advanced caudally to the rostral edge of the lumbar enlargement. Each rat was scalded 5 days after implantation. Two weeks after thermal injury, the agents were injected at a volume of 10 μ l, followed by 10 μ l of saline flush, using a Hamilton glass syringe. No rat received the same doses of drug twice. The following drugs were used: 10 μ g of dizocilpine maleate (MK801) (RBI, MA, USA) as NMDA receptor antagonist, 4 μ g of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (RBI) as AMPA/kinate receptor antagonist and 30 μ g of [N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide] (NS-398) (Alexis, CA,

USA) as cyclooxygenase-2 (COX-2) inhibitor [24]. Each drug was dissolved with 70% dimethylsulfoxide (DMSO). In addition, NS-398 was administered intraperitoneally at doses of 25 mg/kg. Each animal was confined under a Plexiglas box on a raised wire mesh platform similar to the von Frey test. To avoid tissue damage, failure to respond to 7.37 g filaments terminated the test (cut-off) and 12.5 g was assigned. The preinjection pain threshold was determined in every rat using von Frey filaments before administrations. At every 15 min for 1 hr after administration, rats were examined for pain threshold using the von Frey filaments.

III. Results

During the experimental period, all the rats exhibited no evident infections. Every animal showed a similar time course of healing. Re-epithelialization started at the edge of the wound on day 2 postburn. The granulation tissue grew gradually from the wound margin to the wound center. Re-epithelialization was completed on around day 18 postburn. Free behavior without stress in burn injured rats was not different from that in sham-operated rats after day 3 postburn.

Thermal hyperalgesia

Flinching behaviors evoked by the formalin test were significantly increased in the burn group at 2 weeks after injury (Fig. 1A). In the period from 10 to 60 min after formalin injection which is usually called phase 2 and considered to include neurotransmission via mechano-sensitive c fibers [17], the number of flinches was especially increased in the burn group. Therefore, we compared the averages of flinches in phase 2 between the burn injured and sham-operated rats. No change was observed at 1 week after injury between the groups, however, the number of flinches in the burn group was significantly increased at 2 and 3 weeks (Fig. 1B).

Mechanical hyperalgesia

Mechano-hyperalgesia was present after 5–22 days in the burn group (Fig. 2A) tested by von Frey hair filament 4.93. Nociceptive behaviors were significantly increased around 2 weeks after injury. However, mechano-allodynia tested by the von Frey hair filaments 4.13 was not observed in any days examined until 28 days after burn injury (Fig. 2B).

Cold allodynia

Both burn injured and sham operated rats did not exhibit apparent cold allodynia. In both groups, a percent maximum possible response was below 25% throughout the study (0–28 postburn days). There was no significant change between the two groups (Fig. 2C).

Immunohistochemistry

c-Fos expression

c-Fos positive neurons were observed in both the superficial (laminae I, II) and deep layers (III–VI) of the left dor-

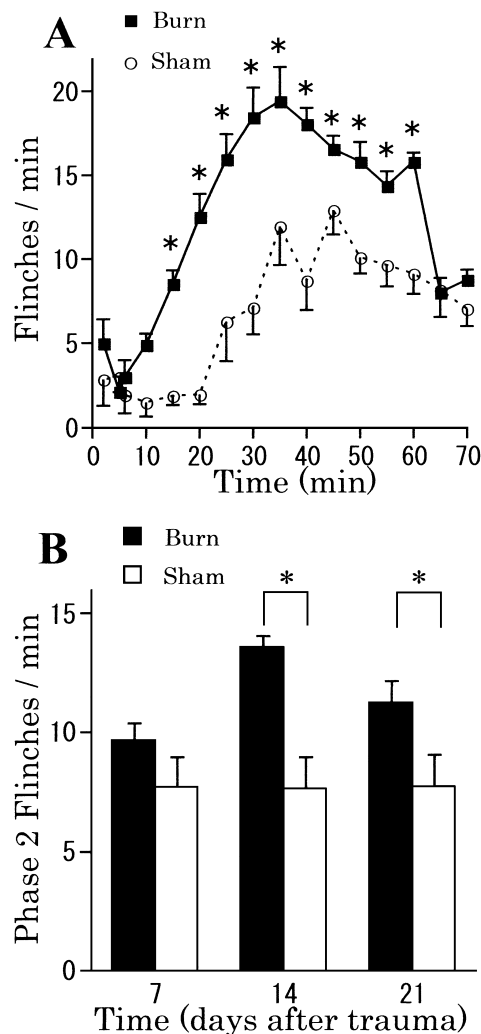


Fig. 1. Effects of injection of 5% formalin into the plantar surface of the left hind paw on flinching behavior. **A:** The number of flinching behaviors in the burn group on the postburn day 14 was significantly increased during phase 2 which was from 10 to 60 min after injection. **B:** Histogram shows the average number of flinches during phase 2 in both groups. At 1 week after injury, no change was observed, but at 2 and 3 weeks in the burn group the number of flinches is significantly increased. Error bars indicate SE. The number of animals used was 6 in each group. (**A, B:** * $p < 0.05$, one-way ANOVA with Scheffé's multiple comparison test)

sal horn (injected site) 1 hr after the 5% formalin injection. There were fewer observed in deep layers than in superficial layers (Fig. 3A). Nucleus in the cell was stained brown by c-Fos antiserum (Fig. 3B). c-Fos positive cells were not detected in other deep layers. The number of c-Fos positive neurons in the left dorsal horn (laminae I–VI) was significantly increased in the burn group compared with that in the sham-operated group (Fig. 3C). In the right dorsal horn, which was the control side, the number of c-Fos positive neurons did not show a significant change between the two groups (data not shown).

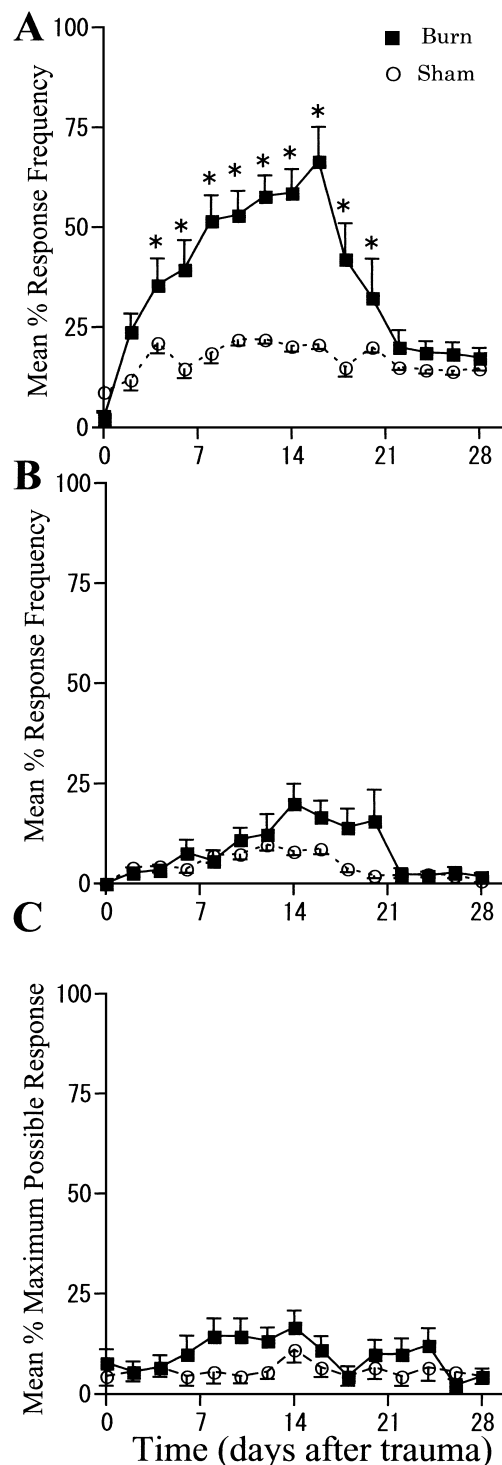


Fig. 2. Changes in mean % response frequency produced by von Frey filaments of 4.93 (**A**) and 4.13 (**B**) and cold allodynia (**C**) by acetone in the foot. X-axis presents the postburn days (Zero indicates the day of burn injury). **A:** In the burn group, mechanical hyperalgesia in the foot is present at 5–22 days. **B:** Mechanical allodynia is not present. **C:** Cold allodynia is not present. Nociceptive behaviors in both feet are markedly increased around 2 weeks after injury. $n = 6$. (* $p < 0.05$, the non-parametric Mann-Whitney U test)

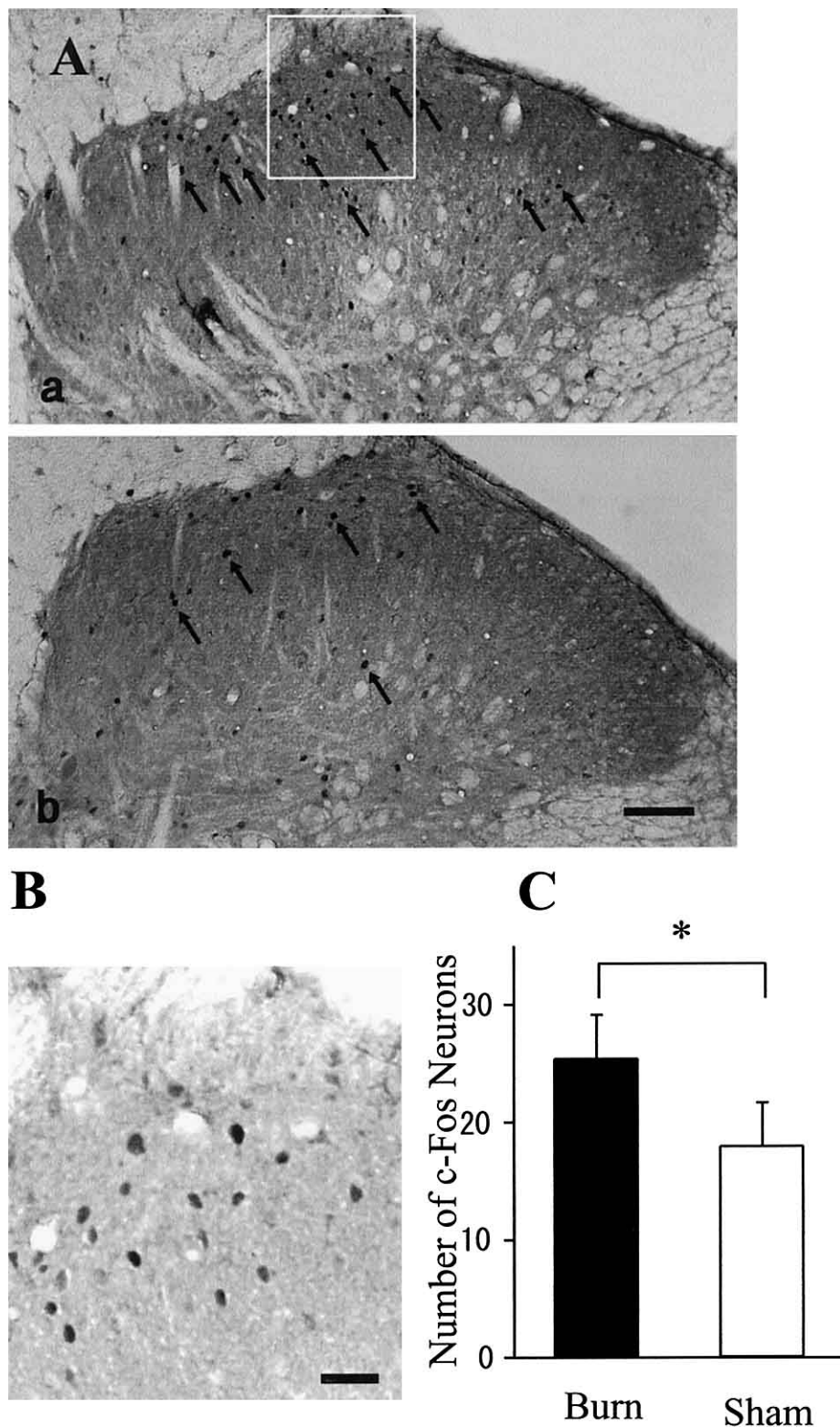


Fig. 3. **A:** Light micrographs show c-Fos immunoreactive neurons in the left dorsal horn (formalin injected site) at spinal cord L4 of burn injured and sham-operated rats on day 14 postburn. **a,** Burn group; **b,** Sham group. The c-Fos positive cells are dominantly expressed in the superficial layer and fewer are expressed in the deep layer. The number of c-Fos positive cells is significantly increased in the burn group compared with the sham-operated group (arrows). Bar=100 μ m. **B:** Light micrograph showing enlarged square in **A**. Bar=20 μ m. **C:** The number of c-Fos positive cells per slices in the burn group is significantly larger than the sham group. $n=5$ (* $p<0.05$, t-test)

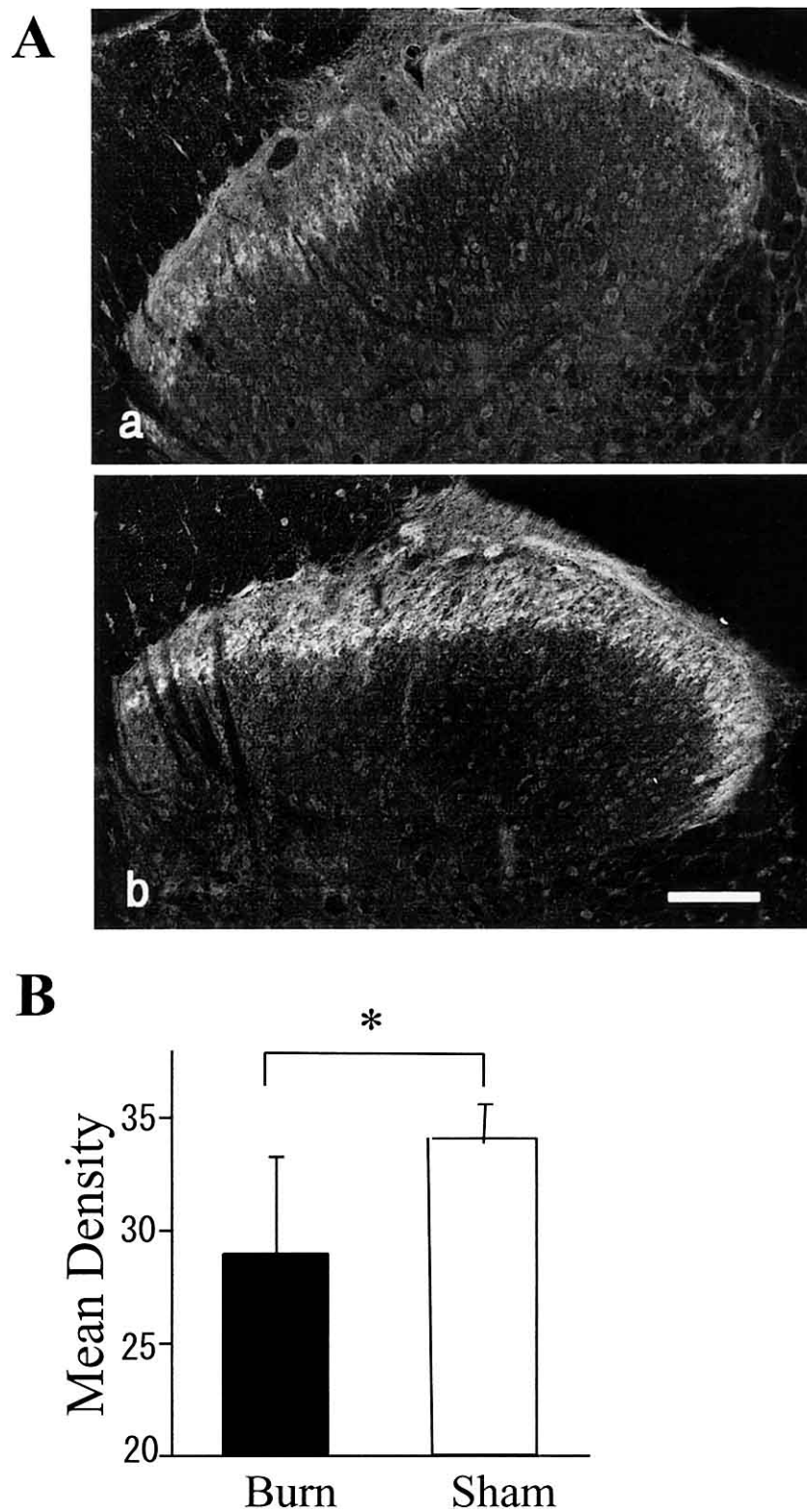


Fig. 4. **A:** Light micrographs show the μ -opioid receptor (MOR) immunoreactive neurons and fibers in the superficial layers of the left dorsal horn at spinal cord L4 of burn injured (**a**) and sham-operated (**b**) rats on day 14 postburn (dark fields). **a**, Burn group; **b**, Sham group. MOR immunoreactivity in the layer (II) of the burn group is weaker and the immunoreactive area is narrower than the sham group. Bar=100 μ m. **B:** Histogram of the quantified MOR immunoreactivity shows a decreased density in the burn group. $n=5$ (* $p<0.05$, t-test)

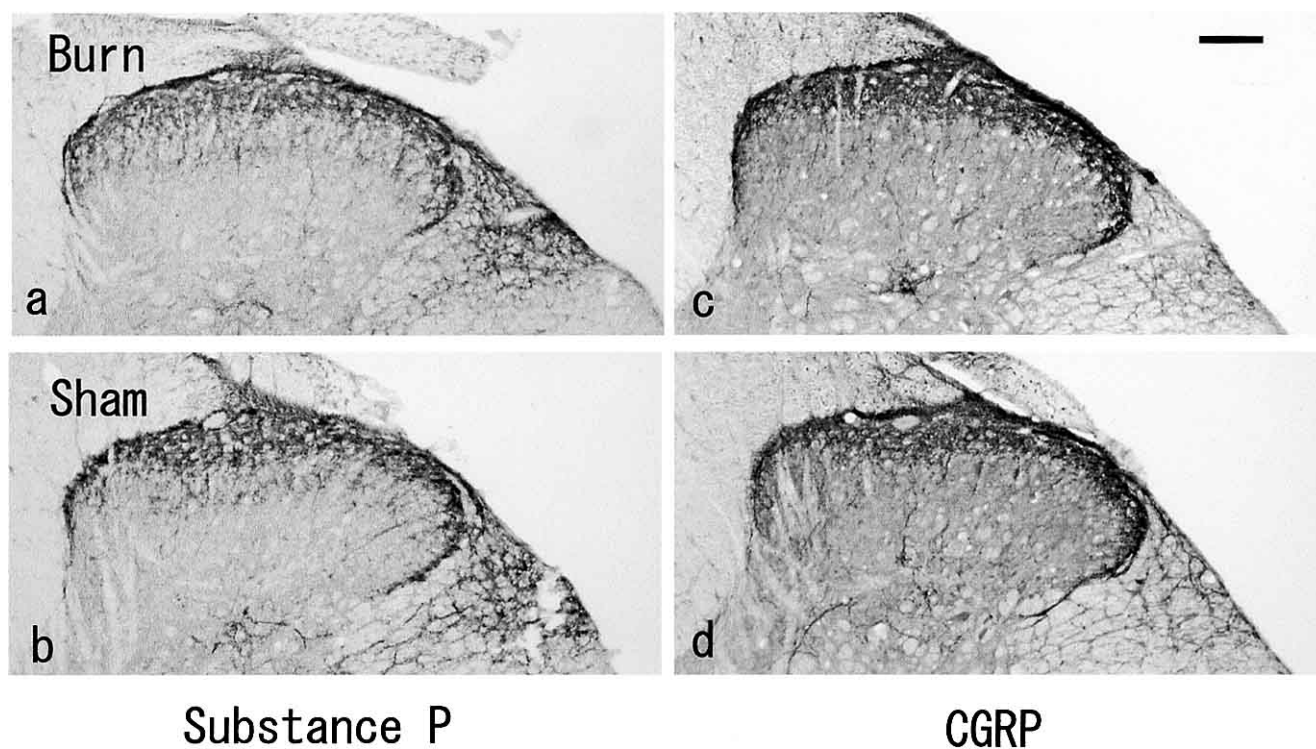


Fig. 5. Light micrographs show dense substance P (a, b) and CGRP (c, d) immunoreactive fibers in the superficial layers of the left dorsal horn at spinal cord L4 of burn injured (a, c) and sham-operated (b, d) rats on day 14 postburn. Bar=100 μ m. Both substance P and CGRP presented similar stainings between the burn and sham group.

MOR expression

Immunoreactivity of MOR was observed in neurons and afferent fibers of the superficial layer (laminae I–II) of the dorsal horn. It was shown to be weaker in the burn group than in the sham group (Fig. 4A). Immunoreactivity of both fibers and cell bodies appeared to decrease in the superficial layer. The quantified MOR immunoreactivity using the NIH image showed a decreased density of laminae I–II in the burn group compared with the shams at 2 weeks after burn injury (Fig. 4B).

Substance P and CGRP expression

Dense substance P immunoreactive (Fig. 5a, b) and CGRP immunoreactive fibers (Fig. 5c, d) were dominantly observed in the superficial layer (laminae I and II) in the dorsal horn of the lumbar spinal cord. These fibers are considered to be coming from mainly small dorsal root ganglion neurons. However, both substance P and CGRP presented similar stainings between the burn and the sham group.

Intrathecal administration

At 2 weeks after thermal injury when all rats developed mechano-hyperalgesia by testing with von Frey hair 4.93, we performed intrathecal administration of NMDA, non-NMDA receptor antagonists or COX-2 inhibitor. Intrathecal injection of 70% DMSO as a control did not show any changes in pain threshold. In the burn group, injection of MK801 (Fig. 6A) and CNQX (Fig. 6B) transiently increased

the pain threshold of the foot almost to the levels of the sham group and then gradually decreased. The von Frey threshold in MK801 or CNQX injected rats returned to the preinjection levels, that is mechano-hyperalgesia at 1 hr after injection.

However, neither intrathecal nor intraperitoneal injection of NS-398 recovered the pain threshold (Fig. 6C).

IV. Discussion

In the present study we found foot hyperalgesia after burn injury at the thoracic area during the recovery stage from 5 to 22 days. We observed thermal hyperalgesia using the formalin test and mechano-hyperalgesia using the von Frey test. Hyperalgesia was not observed in the acute period until 5 days after the burn. Both tests showed a peak hyperalgesia at 2 weeks after injury. In the formalin test, since phase 2 activity is considered to reflect the development of a central facilitation [17], burned animals showing an increase seemed to develop the facilitation of pain at the spinal cord. In the von Frey test, the 12 mN filament is within the force range that activates low-threshold A- and C-mechanoreceptors, while the 47-mN filament can also activate nociceptors [18]. Therefore the present model is considered to develop mechano-hyperalgesia but not mechano-allodynia.

We confirmed a histological change occurred in the burn group at the dorsal horn of the lumbar spinal cord where c-Fos positive cells were increased by plantar forma-

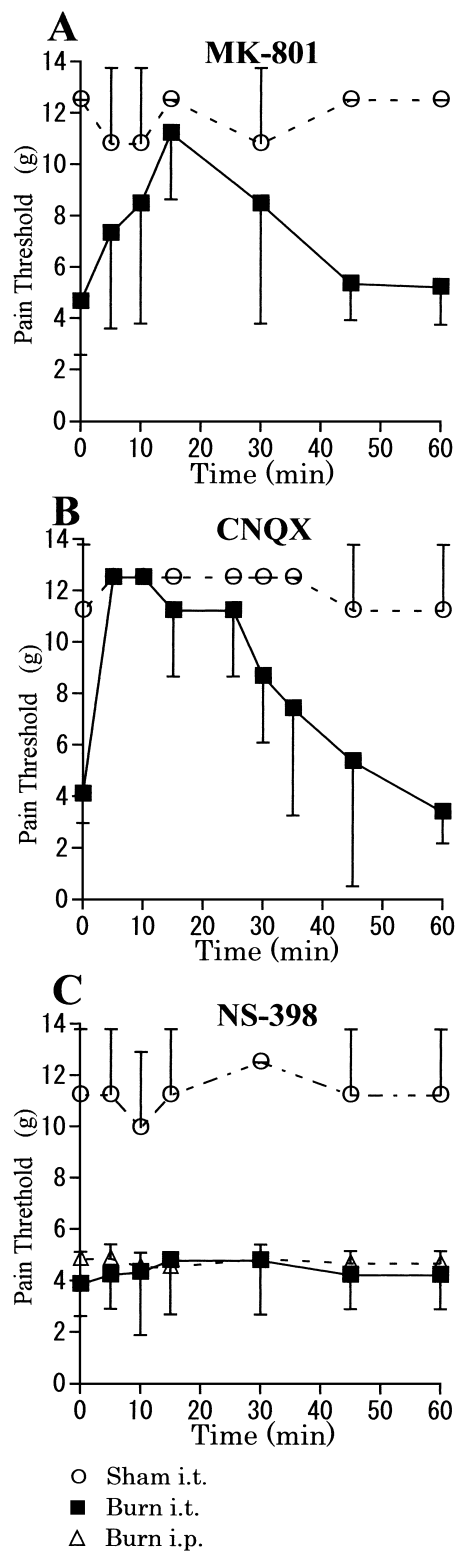


Fig. 6. Changes in pain threshold by von Frey hair 4.93 after intrathecal administration of MK801 (A), CNQX (B) and NS-398 (C) at 2 weeks after injury. Mechanical hyperalgesia is present in the burn group. Intrathecal administration of MK801 and CNQX reversed the pain threshold transiently up to the control level. However, intrathecal and intraperitoneal injection of NS-398 did not alter the pain threshold. $n=4$.

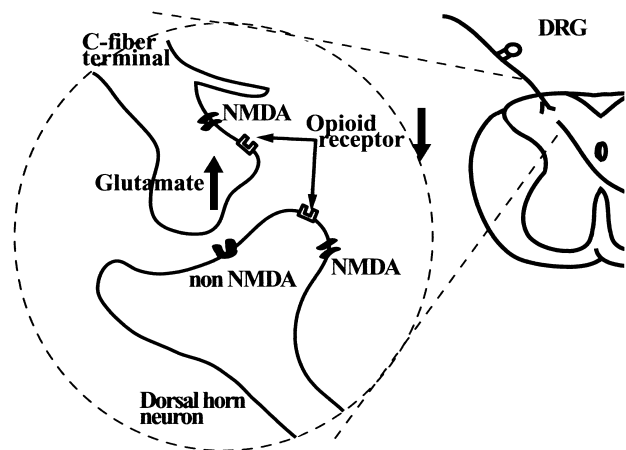


Fig. 7. The schematic representation at the lumbar spinal cord in this model. Sensitivity of postsynaptic dorsal horn neurons or functional release of presynaptic terminals at the spinal cord seemed to be affected in the burned animals. Consequently glutaminergic neurotransmission was increased via NMDA and/or non NMDA receptor. A downregulation of μ -opioid receptor expression also participated in this phenomenon.

lin injection. The proto-oncogene *c-fos* is rapidly and transiently expressed in response to noxious inputs in the central nervous system, including the spinal cord and *c-Fos* protein has been extensively applied as a marker of neuronal activation in the dorsal horn neurons following stimulation [5, 22]. In the present model, glutamate as an extracellular signal appears to be involved in inducing *c-Fos* expression after formalin injection into the hindpaw since MK801 block can inhibit *c-Fos* expression induced by noxious stimuli at the spinal cord level [9]. It is also reported that *c-Fos* is expressed in NMDA receptor-containing neurons in the dorsal horn of the spinal cord [25].

Furthermore, experiments using intrathecal administration of MK801, CNQX and NS-398 showed that glutamate receptor antagonist but not COX-2 inhibitor recovered the threshold to the control levels by the von Frey test to the hindpaw. All these findings indicate glutamate neurotransmission or its receptor sensitivity at the spinal cord level was increased in the burn model.

Cytokines such as interleukin-6, tumor necrosis factor- α , which are increased in inflammation, were reported to increase in plasma and tissues for 24 hr after 20% BSA with third degree-thermal injury [8]. Prostaglandins and COX-2 are also known to have roles in inflammation and pain [2, 19]. However, in the present model, involvement of COX-2 in hyperalgesia due to active inflammation is unlikely during the healing stage at 2 weeks after injury because neither intraperitoneal nor intrathecal injection of COX-2 inhibitor affected the increased threshold of foot hyperalgesia.

We also observed MOR immunoreactivity decreased compared with control rats. The reason for down-regulation of MOR at the dorsal horn is unclear. Increased plasma opioids reported during the acute period of burn injury [15, 21] may downregulate the receptor level. This decreased

MOR expression was also suggested to be involved in the development of hyperalgesia due to the decrease in the inhibitory regulation of nociceptive neurotransmission pre- and post-synaptically in the burn model. The schematic representation at the lumbar spinal cord in this model was shown in Fig. 7.

In the present model, we did not detect any significant changes in the expressions of neuropeptides such as substance P or CGRP in the lumbar dorsal horn and dorsal root ganglion by peptide immunocytochemistry. Both substance P and CGRP exist dominantly in small sensory neurons and particularly substance P is considered to deliver nociceptive information [4]. These results suggest the peripheral dorsal ganglion neurons in the lower limb did not change so much regarding pain transmission. It is more likely that the sensitivity of postsynaptic dorsal horn neurons or the release of neurotransmitters such as glutamate from the presynaptic terminals at the spinal cord was affected in the burned animals.

Reduced expression of MOR in the dorsal horn was also observed at the level of the thoracic spinal cord (data not shown). Taken together, it was suggested that central hyperalgesia at the total spinal cord was developed in burn injured animals.

The mechanism to connect the thoracic healing skin from burn injury and lumbar spinal hyperalgesia remains to be clarified. One candidate to explain this mechanism is a nerve growth factor (NGF) because regenerating skin from the wound was known to increase NGF [12] and overproduction of NGF in the skin by attaching the NGF gene after the K14 keratin promoter in the transgenic mouse exhibited a mechano-hyperalgesia [1]. Moreover, rats were reported to develop a profound hypersensitivity to noxious heat and mechanical stimuli but not allodynia probably due to the central mechanisms after only a single systemic injection of NGF [10, 11]. The burn model rats in the present study also developed mechanical and thermal hyperalgesia but not allodynia.

The involvement of NGF in this model will be proven in the future study.

In conclusion, we found foot hyperalgesia during the recovery stage of thoracic thermal injured rats. From this study, we may insist that patients who suffered large burn injuries should be nursed taking care not only of damaged skin but also of non-injured areas.

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