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Mechanical Pain Hypersensitivity after Incisional Surgery Is Enhanced in Rats Subjected to Neonatal Peripheral Inflammation

Effects of N-*Methyl-*D*-aspartate Receptor Antagonists*

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Background: **Neonatal pain and inflammation may lead to a long-term effect on nociceptive processing in adults. The current study examined the characteristics of postoperative incisional pain behaviors in adult rats that were subjected to neonatal peripheral inflammation.**

Methods: **Rat pups received a subcutaneous injection of saline or carrageenan into the plantar surface of the left hind paw at postnatal day 1. Naive pups were used as the control. Paw withdrawal thresholds to punctuate mechanical stimuli were examined at postnatal days 35, 42, and 49. After rats received a plantar incision on the left or right hind paw at postnatal day 50, paw withdrawal thresholds were measured at 4 h, 1 day, 2 days, 3 days, 5 days, and 7 days after incision. In addition, spinal cord Fos expression was detected at 2 h after incision. Finally, the effects of intrathecal *N*-methyl-D-aspartate receptor antagonists DL-2-amino-5-phosphonovaleric acid and dizocilpine and the nitric oxide synthase inhibitor L-N^G-nitro-arginine methylester on incisional pain were examined at 4 h after incision.**

Results: **Although the rats subjected to neonatal peripheral carrageenan injection developed mechanical hypoalgesia in bilateral hind paws at baseline, they displayed increased spinal cord Fos expression at 2 h and exaggerated mechanical pain hypersensitivity at 4 h (but not at other time points) after plantar incision. Intrathecal DL-2-amino-5-phosphonovaleric acid, dizocilpine, and L-N^G-nitro-arginine methylester significantly attenuated incision-induced mechanical pain hypersensitivity at 4 h after incision in the neonatally carrageenan-treated rats, but not in the naive or neonatally saline-treated rats.**

Conclusions: **The authors' results suggest that early inflammatory insults during the neonatal period could produce excessive incision-associated mechanical pain hypersensitivity in adult rats. Spinal cord *N*-methyl-D-aspartate receptors and downstream nitric oxide signaling might contribute to this abnormal pain hypersensitivity, although the mechanisms underlying the long-term effect of neonatal inflammation are still unclear.**

CURRENT medical procedures in neonatal intensive care units have increased the survival of premature and other high-risk neonates. However, these procedures are often invasive and traumatic. They not only cause considerable pain during their application but also are followed by local inflammation that lasts for several hours or even days.[1] Preclinical studies indicate that neonatal inflammatory insults might produce long-term effects on pain responsivity.[2,3] For example, adult rats subjected to a single subcutaneous injection of carrageenan into the plantar surface of a hind paw during the neonatal period (from postnatal days 0 to 5) display bilateral increase in nociceptive threshold (hypoalgesia) at baseline and ipsilateral enhanced increase in nocifensive responses (exaggerated hyperalgesia) to complete Freund adjuvant–induced inflammation.[4–6] Further analysis by DNA microarray and real-time reverse-transcription polymerase chain reaction demonstrated that neonatal local noxious insult altered the expression of multiple genes in the spinal dorsal horn of adult rats.[7] For example, reinflammation of the left hind paw in adult rats induced significant up-regulation in expression of the genes encoding for *N*-methyl-D-aspartate (NMDA) receptor subunits in the ipsilateral dorsal horn in the neonatally carrageenan-treated rats, as compared with that in similarly inflamed animals.[7] Changes in the expression of these genes in the spinal cord are proposed to be involved in the central mechanism that underlies the enhanced pain hypersensitivity produced by an inflammatory insult in the neonatal period, but no direct pharmacologic evidence supports this proposal.

Incisional pain is a common syndrome in many patients postoperatively. Optimizing postoperative pain treatment increases patient comfort, reduces complications, and improves postoperative outcome.[8] Postoperative pain management strategies are mainly based on studies performed in animal models of inflammatory pain.[9,10] However, recent evidence indicates that the neurobiology of postoperative incisional pain may be different from inflammatory and neuropathic pain.[11] It is unknown whether early, local, noxious insults during the neonatal period affect postoperative incisional pain in adults.

In the current study, we characterized mechanical pain hypersensitivity induced by incision injury in adult rats that were subjected to a subcutaneous injection of carrageenan into a hind paw at postnatal day 1. We also examined whether spinal NMDA receptors and down-

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stream nitric oxide signaling contributed to the excessive incision-associated mechanical pain hypersensitivity in these rats.

Materials and Methods

Animals

Rats used in this study were born from time-pregnant Sprague-Dawley dames purchased from Yang Ming University Animal Facilities (Taipei, Taiwan, Republic of China). The animals arrived at the Taipei Veterans General Hospital (Taipei, Taiwan, Republic of China) on the 16th day of pregnancy and were allowed to deliver at term. They were housed individually in cages with food and water available *ad libitum* and kept on a standard 12 h–12 h light–dark cycle. The sexing of the pups was identified at birth by evaluation of the anogenital distance. Males were returned to the mother, and females were killed. After weaning at postnatal day 21, the male pups were housed in groups of three until the end of the experiments, except that rats with intrathecal catheter placement were housed individually. The animals were used in accordance with protocols approved by the Animal Care and Use Committees at the Taipei Veterans General Hospital and were consistent with the ethical guidelines of the National Institutes of Health and the International Association for the Study of Pain. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Experimental Drugs

Isoflurane was purchased from Baxter Caribe Inc. (Guayama, Puerto Rico). Carrageenan, DL-2-amino-5-phosphonovaleric acid (DL-APV), dizocilpine (MK-801), and L-N^G-nitro-arginine methylester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in 0.9% physiologic saline. All solutions were freshly prepared on the day of the experiments. The doses of drugs used were based on the previous studies.[12,13]

Carrageenan Injection

Neonatal pups were divided into three groups: naive, saline treated, and carrageenan treated. In the carrageenan-treated group, the pups received a single injection of 0.25% carrageenan (1 μl/g) into the plantar surface of the left hind paw at postnatal day 1. In the saline-treated group, the pups received an equal volume of saline injection. Behavioral testing as described below was performed on postnatal days 35, 42, and 49.

Behavioral Testing

Mechanical pain hypersensitivity was assessed by studying the 50% paw withdrawal threshold to a tactile stimulus with calibrated von Frey monofilaments. Paw withdrawal thresholds were measured using the up–down method, as previously described.[14] Briefly, rats were placed in a transparent plastic box with a metal wire mesh floor and were allowed to acclimate to the test chamber for 1 h. Von Frey filaments with approximately equal logarithmic incremental (0.22) von Frey values (1.57, 2.45, 4.21, 7.15, 12.64, 21.17, 37.04, 61.45, 101.92, and 159.74 in mN, starting from 21.17 mN) were used to determine the threshold stiffness required for 50% paw withdrawal. Each von Frey filament was presented perpendicularly, pointing distally toward the incision 1–2 mm into the foot pad and held in this position for approximately 3–5 s, with enough force to cause a slight bend in the filament. The next filament was applied approximately 5–10 s later. Positive responses included an abrupt withdrawal of the hind paw from the stimulus, or flinching behavior immediately after removal of the stimulus. The 50% threshold value was calculated using the formula of Dixon.[15] All behavioral tests were conducted by a single experimenter and in a blinded manner.

Plantar Incision

At postnatal day 50, rats were anesthetized with 2–3% isoflurane delivered *via* a nose cone. According to the previous studies,[16,17] the plantar aspect of the hind paw was prepared, and a 1-cm longitudinal incision was made through the skin, fascia, and muscle of the plantar aspect of the hind paw. The skin was apposed with two 5-0 nylon sutures, and the wound site was covered with antibiotic ointment. The rats were allowed 1 h for complete recovery from inhaled anesthesia. Behavioral testing as described above was performed at 4 h, 1 day, 2 days, 3 days, 5 days, and 7 days after incision. Sutures were removed at the end of postoperative day 2.

Intrathecal Catheter Placement and Drug Injection

Animals under isoflurane anesthesia were implanted with an intrathecal catheter. A polyethylene (PE-10) tube was inserted into the subarachnoid space at the rostral level of the spinal cord lumbar enlargement segments through an incision at the atlanto-occipital membrane, according to the previously described method.[13,18,19] The animals were allowed to recover for 5–10 days before incisional surgery was performed. Rats that showed any neurologic deficits postoperatively were discarded from the study. PE-10 catheter position from each animal was confirmed after behavioral testing.

After behavioral testing at 4 h after incision, vehicle (saline, 10 μl), MK-801 (a noncompetitive NMDA receptor antagonist, 40 nmol/10 μl), DL-APV (a competitive NMDA receptor antagonist, 10 and 30 nmol/10 μl), or L-NAME (a nonselective nitric oxide inhibitor; 200 nmol/10 μl) was administered intrathecally. Behavioral testing as described above was conducted at 20, 40, 60, 80, 100, 120, 180, and 240 min after injection.

Locomotor Function Testing

To examine whether the drugs used in behavioral testing affected the locomotor function, the placing reflex was tested as described previously.[17] In brief, the animals received intrathecal injection with vehicle (saline), MK-801 (40 nmol/10 μl), DL-APV (10 nmol and 30 nmol/10 μl), or L-NAME (200 nmol/10 μl). The placing reflex evaluation was performed at 20, 40, 60, 80, 100, 120, 180, and 240 min after injection. Each rat was placed on a table, and the dorsum of either hind paw was drawn across the edge of the table. The score for placing reflex was based on the latency of each reflex exhibited in three trials (2 = normal [< 1 s]; 1 = delay [1–2 s]; 0 = defect [> 2 s]). Rat general behaviors, including spontaneous activity, were also observed.

Fos Immunocytochemistry

The rats were deeply anesthetized with isoflurane and perfused with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) at 2 h after plantar incision. The fourth and fifth lumbar segments were harvested, postfixed in the same fixative solution for 4–8 h, cryoprotected by immersing in 30% sucrose overnight at 4°C, and frozen-sectioned at 25 μm. Sections were processed for Fos immunocytochemistry *via* the conventional avidin–biotin complex method based on previous studies.[20,21] Briefly, sections were incubated in polyclonal rabbit anti-Fos antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 0.01 M phosphate-buffered saline (pH 7.4) containing 3% normal goat serum and 0.3% Triton X-100 for 48 h at 4°C. The sections were then incubated in biotinylated goat anti-rabbit immunoglobulin G (1:200; Vector Laboratories, Burlingame, CA) for 1 h at room temperature followed by avidin–biotin–peroxidase complex (1:100; Vector Laboratories) for 1 h at 37°C. The immune reaction product was visualized by catalysis of 3,3-diaminobenzidine by horseradish peroxidase in the presence of 0.01% H_2O_2.

Ten sections were randomly taken from the fourth and fifth lumbar segments of the spinal cord of each rat and examined with a microscope linked to a computer-assisted image-processing system through a video camera. To quantify the anatomical results, the spinal dorsal horn was divided into three regions: the superficial laminae (laminae I and II), the nucleus proprius (laminae III and IV), and the neck of the dorsal horn (laminae V and IX). The number of Fos immunoreactive neurons was independently counted by two experimenters, the results of which were consistent within 10%.

Statistical Analysis

Data were presented as median ± interquartile range or mean ± SEM, when appropriate. Mechanical withdrawal thresholds were compared using nonparametric analyses. The Friedman test for within-group and the Kruskal–Wallis and Mann–Whitney rank sum tests for between-group comparisons were used. Multiple comparisons after Friedman and the Kruskal–Wallis tests were performed using a two-tailed Dunn tests. Analgesic indexes for the effects of drugs were represented by the area under the curve, calculated for each rat by the trapezoidal method.[22] Indexes were compared by the Kruskal–Wallis test and Dunn *post hoc* test. The number of spinal Fos-like immunoreactive neurons was compared by one-way analysis of variance and Student–Newman–Keuls *post hoc* comparisons. Significance was set at $P < 0.05$. All statistical analyses were performed using the SigmaStat (Systat, Port Richmond, CA) statistical software package.

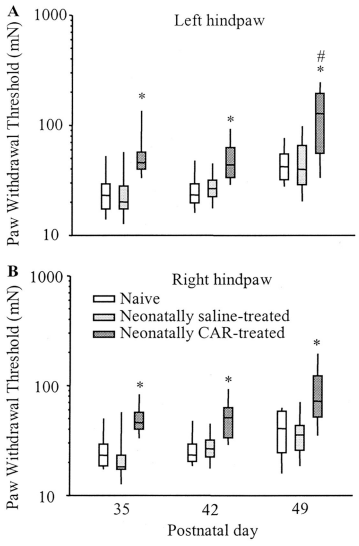

Fig. 1. Box plot representing paw withdrawal thresholds in response to mechanical stimuli on the left (*A*) and right (*B*) hind paws in naive (n = 12), neonatally saline-treated (n = 12), and neonatally carrageenan (CAR)-treated (n = 12) groups at postnatal days 35, 42, and 49. The box and whisker plots are expressed as medians (*horizontal lines*) with first and third quartiles (*boxes*), and 10th and 90th percentiles (*vertical lines*). * $P < 0.05$ *versus* the corresponding naive and the neonatally saline-treated groups at each time point. # $P < 0.05$ *versus* the neonatally CAR-treated groups at postnatal days 35 and 42.

Results

Development of Basal Hypoalgesia in the Neonatally Carrageenan-treated Rats

Consistent with previous studies,[4,5] adult rats that received carrageenan injection into the left hind paw at postnatal day 1 exhibited basal mechanical hypoalgesia on both hind paws when paw withdrawal thresholds in response to punctuate mechanical stimuli were measured at postnatal days 35, 42, and 49. Basal median withdrawal thresholds in the neonatally carrageenan-treated group (n = 12) were significantly increased, compared with those recorded for the corresponding hind paw in naive (n = 12) or the neonatally saline-treated (n = 12) rats at these three time points (Kruskal–Wallis test, $df = 2$, $P < 0.05$; figs. 1A and B). The highest

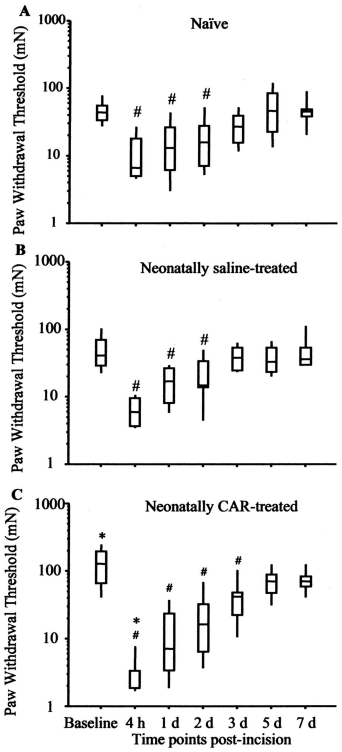

Fig. 2. Paw withdrawal thresholds in response to mechanical stimuli on the left hind paw at postnatal day 49 (baseline) and at 4 h, 1 day, 2 days, 3 days, 5 days, and 7 days after plantar incision of the left hind paw in naive (n = 12) (*A*), neonatally saline-treated (n = 12) (*B*), and neonatally carrageenan (CAR)-treated (n = 12) (*C*) groups. # $P < 0.05$ *versus* the corresponding baseline in each group. * $P < 0.05$ *versus* the corresponding time points in the naive and saline-treated groups.

increase in basal median mechanical withdrawal threshold was observed at postnatal day 49 (fig. 1). On the left hind paw, median withdrawal threshold was 41.9 mN for the naive group, 39.7 mN for the neonatally saline-treated group, and 126.8 mN for the neonatally carrageenan-treated group on postnatal day 49 (Kruskal–Wallis test, $df = 2$, $P < 0.001$; fig. 1A). On the right hind paw, median withdrawal threshold was 40.5 mN for the naive group, 35.4 mN for the neonatally saline-treated group, and 71.8 mN for the neonatally carrageenan-treated group on postnatal day 49 (Kruskal–Wallis test, $df = 2$, $P < 0.01$; fig. 1B).

Enhanced Plantar Incision–induced Mechanical Pain Hypersensitivity in the Neonatally Carrageenan-treated Rats

After plantar incision on the left hind paw, mechanical pain hypersensitivity (*i.e.*, the marked decreases in paw withdrawal thresholds in response to punctuate mechanical stimuli) was developed on the ipsilateral side in the naive (Friedman test, $\chi^2_6 = 37.2$, n = 12, $P < 0.001$; fig. 2A), neonatally saline-treated (Friedman test, $\chi^2_6 = 34.9$, n = 12, $P < 0.001$; fig. 2B), and neonatally carrageenan-treated (Friedman test, $\chi^2_6 = 54.8$, n = 12, $P < 0.001$; fig. 2C) groups. It reached a peak at 4 h, lasted for 2 days after incision in the naive and neonatally saline-treated rats, and persisted for 3 days after incision in the neonatally carrageenan-treated group. More importantly, the median mechanical withdrawal threshold (1.86 mN) in the neonatally carrageenan-treated group was significantly lower compared with that in the naive group (6.57 mN) or in the neonatally saline-treated group (5.93 mN) at 4 h (Kruskal–Wallis test, $df = 2$, $P < 0.001$), but not at days 1 and 2 after incision (Kruskal–Wallis test, $df = 2$, $P > 0.05$; fig. 3A). In contrast, after plantar incision on the left hind paw, the median mechanical withdrawal threshold on the right hind paw in the neonatally carrageenan-treated group was not significantly different from that in either the naive or neonatally saline-treated group (Kruskal–Wallis test, $df = 2$, $P > 0.05$; fig. 3B) or from the corresponding baseline (Kruskal–Wallis test, $df = 2$, $P > 0.05$; fig. 3B).

To test whether incisional surgery on the neonatally intact hind paw affected mechanical withdrawal thresholds, we performed plantar incision on the right hind paw of three animal groups. Median mechanical withdrawal threshold on the right hind paw in the neonatally carrageenan-treated group (n = 12) was similar to that in naive group (n = 12) (data not shown) and to that in the neonatally saline-treated group (n = 12) (Mann–Whitney test, $P > 0.05$; fig. 3C), although mechanical withdrawal thresholds on the right side in all three groups were significantly decreased after plantar incision (figs. 1B and 3C). As expected, after plantar incision on the right hind paw, the median mechanical withdrawal threshold on the left hind paw in the neonatally carrageenan-treated group was not significantly different from that in either the naive (data not shown) or the neonatally saline-treated group (Mann–Whitney test, $P > 0.05$; fig. 3D). Interestingly, the median mechanical withdrawal threshold on the left hind paw in the neonatally carrageenan-treated group at 4 h (Mann–Whitney test, $P < 0.05$; fig. 3D), but not at days 1 and 2 (Mann–Whitney test, $P > 0.05$; data not shown), after an incision of the right hind paw was markedly reduced compared with the corresponding baseline.

Increased Plantar Incision–induced Spinal Dorsal Horn Fos Expression in the Neonatally Carrageenan-treated Rats

Fos as the protein product of *c-fos*, a proto-oncogene, has been widely used as a marker for functional activity of the neurons, especially in the study of the response of the spinal dorsal horn neurons to noxious stimulation.[23] Its expression could be specifically induced in the superficial and deep dorsal horn and significantly attenuated by NMDA receptor antagonists and nitric oxide synthase inhibitors.[20,21,24–27] To further examine whether neonatal carrageenan injection affected incision-induced functional activity in spinal dorsal horn neurons of the adult rats, we measured Fos expression in the dorsal horn of the three groups after plantar incision. Consistent with previous

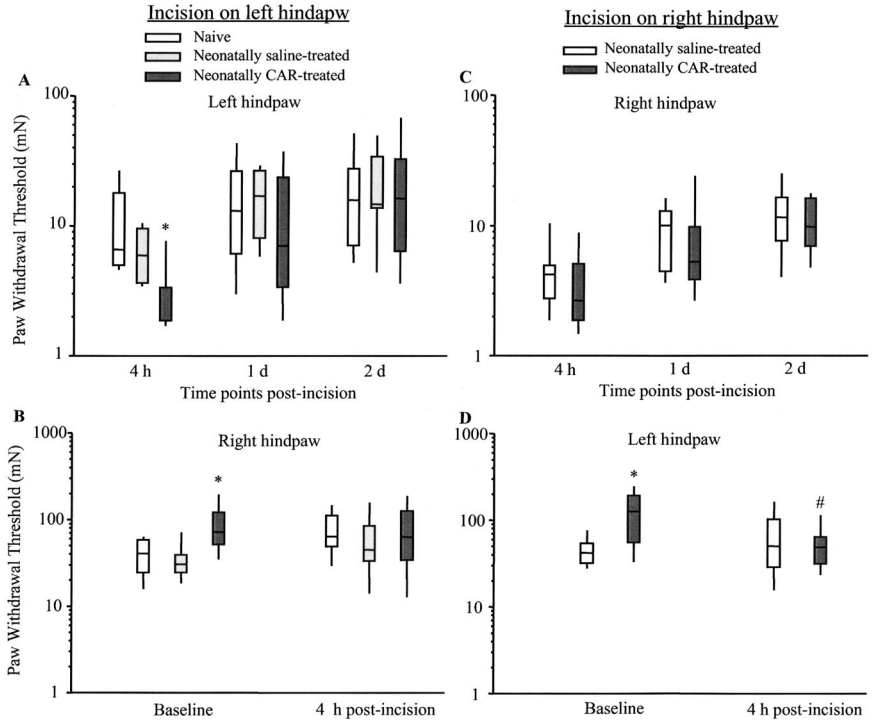

Fig. 3. Effect of incision on the left or right hind paw on mechanical withdrawal thresholds in naive, neonatally saline-treated, and neonatally carrageenan (CAR)-treated groups. (*A* and *B*) Paw withdrawal thresholds in response to mechanical stimuli on the left hind paw at 4 h, 1 day, and 2 days after incision of the left hind paw (*A*), and on the right hind paw before incision (baseline) and at 4 h after incision of the left hind paw (*B*) in naive (n = 12), neonatally saline-treated (n = 12), and neonatally CAR-treated (n = 12) groups. (*C* and *D*) Paw withdrawal thresholds in response to mechanical stimuli on the right hind paw at 4 h, 1 day, and 2 days after incision of the right hind paw (*C*), and on the left hind paw before incision (baseline) and at 4 h after incision of the right hind paw (*D*) in neonatally saline-treated (n = 12) and neonatally CAR-treated (n = 12) groups. * *P* < 0.05 *versus* either the naive or saline-treated groups at the same time point. # *P* < 0.05 *versus* the neonatally CAR-treated group at baseline.

studies in the formalin model,[20,21] plantar incision induced Fos expression in the ipsilateral dorsal horn of spinal cord in the naive (n = 5), neonatally saline-treated (n = 5), and neonatally carrageenan-treated (n = 5) groups (figs. 4A and B). Most of the Fos-like immunoreactive neurons were distributed in the medial part of the superficial laminae (laminae I and II), and a few were observed in laminae III and VI. As expected, without plantar incision, little or no Fos expression was detected in the dorsal horns of the three groups (fig. 4C). Our pilot work showed that incision-induced spinal cord Fos expression was time dependent and reached a peak at approximately 2 h after incision. In the current study, we quantified Fos expression at 2 h and found that the number of incision-induced Fos-like immunoreactive neurons in laminae I and II and laminae III and IV in the neonatally carrageenan-treated rats was significantly increased, compared with the naive or the neonatally saline-treated rats (figs. 4A, B, and D). In laminae I and II, the average number of Fos-like immunoreactive neurons per section was 41 ± 2.89 for the naive group, 37.3 ± 3.84

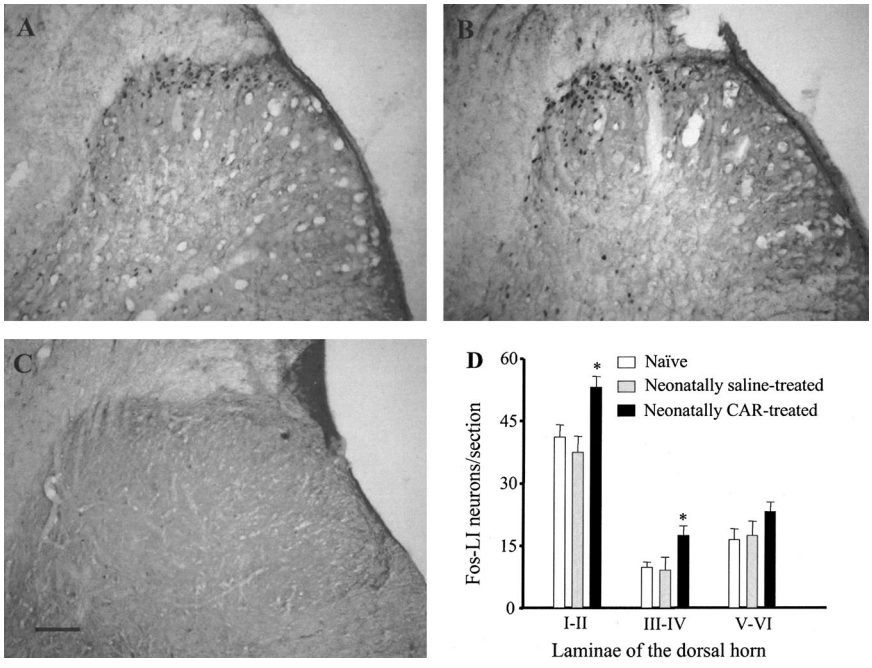

Fig. 4. Representative photomicrographs illustrating the distribution of Fos-like immunoreactive (LI) neurons in the ipsilateral dorsal horn of the fifth lumbar spinal segment at 2 h after plantar incision of the left hind paw in neonatally saline-treated (*A*) and carrageenan (CAR)-treated (*B*) groups. Without plantar incision, little or no Fos expression was observed in the dorsal horn in the naive (data not shown), the neonatally saline-treated (data not shown), or the neonatally CAR-treated (*C*) groups. (*D*) Quantification of Fos-like immunoreactive neurons in laminae I and II, III and IV, and V and VI. Ten random sections were taken from the fourth and fifth lumbar segments in each rat. n = 5/group. * *P* < 0.05 *versus* the naive or the neonatally saline-treated group. *Scale bar*: 100 μm.

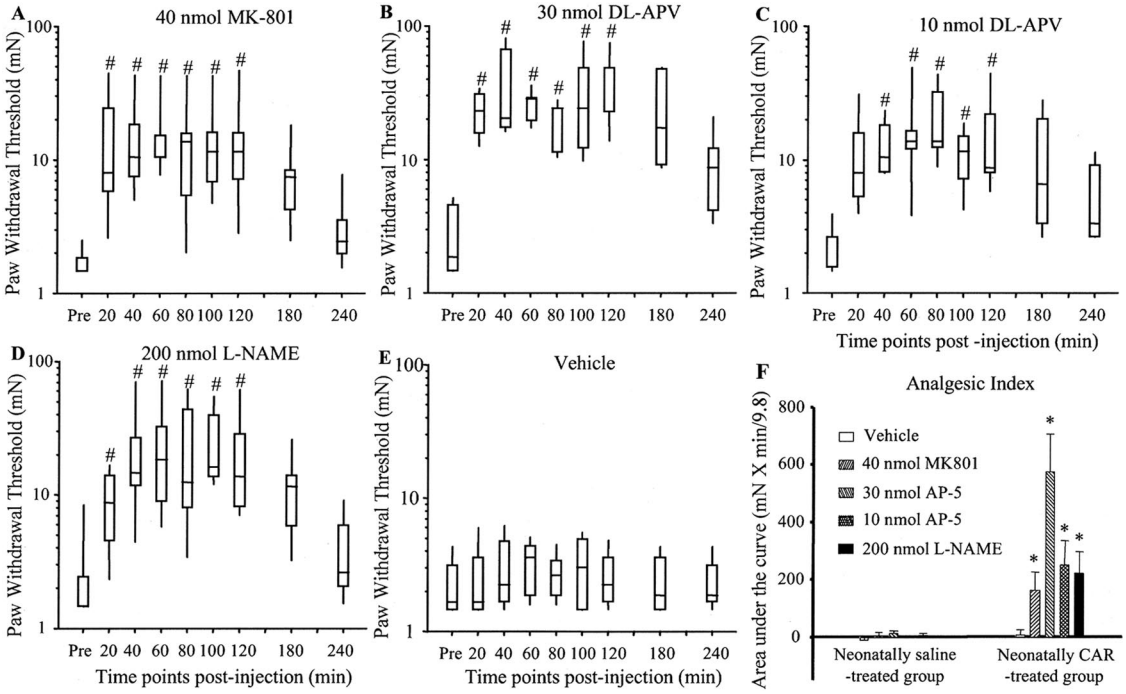

Fig. 5. Effect of intrathecal 40 nmol dizocilpine (MK-801) (n = 7; *A*), 30 nmol DL-2-amino-5-phosphonovaleric acid (DL-APV) (n = 7; *B*), 10 nmol DL-APV (n = 7; *C*), 200 nmol L-N^G-nitro-arginine methylester (L-NAME) (n = 7; *D*), or vehicle (saline) (n = 7; *E*) on incision-induced mechanical pain hypersensitivity at 4 h after incision in neonatally carrageenan (CAR)-treated group. Paw withdrawal thresholds were measured before drug injection (Pre) and at 20, 40, 60, 80, 100, 120, 180, and 240 min after drug injection. # $P < 0.05$ *versus* before drug injection (Pre). (*F*) Analgesic indices of 40 nmol MK-801 (n = 7), 10 nmol DL-APV (n = 7), 30 nmol DL-APV (n = 7), 200 nmol L-NAME (n = 7), and vehicle (n = 7) in the neonatally saline-treated and neonatally CAR-treated groups. * $P < 0.05$ *versus* vehicle.

for the neonatally saline-treated group, and 53 ± 2.52 for the neonatally carrageenan-treated group ($F_{2,12} = 4.89$, $P < 0.05$). In laminae III and IV, the average number of Fos-like immunoreactive neurons per section was 9.67 ± 1.2 for naive group, 9.0 ± 3.1 for the neonatally saline-treated group, and 17.33 ± 2.89 for the neonatally carrageenan-treated group ($F_{2,12} = 9.06$, $P < 0.05$). No difference in number of Fos-like immunoreactive neurons in laminae V and VI was observed among three groups ($F_{2,12} = 1.17$, $P > 0.05$; fig. 4D).

Effects of Intrathecal MK-801, DL-APV, and L-NAME on Plantar Incision–induced Mechanical Pain Hypersensitivity

Consistent with previous studies,[12,17] intrathecal administration of 40 nmol MK-801, 30 nmol DL-APV, or 200 nmol L-NAME at 4 h after incision did not produce significant effects on incision-induced decreases in median mechanical withdrawal thresholds in the naive (data not shown) or the neonatally saline-treated (Friedman test, $\chi^2_8 = 6.945$ for MK-801, $\chi^2_8 = 8.75$ for DL-APV, $\chi^2_8 = 14.36$ for L-NAME, $\chi^2_8 = 10.08$ for vehicle, $P > 0.05$ for all, n = 7/group; data not shown) groups. However, incision-induced decrease of median mechanical withdrawal threshold in the neonatally carrageenan-treated group was significantly reversed by intrathecal 40 nmol MK-801 (Friedman test, $\chi^2_8 = 32.4$, n = 7, $P < 0.001$; fig. 5A), 30 nmol DL-APV (Friedman test, $\chi^2_8 = 34.1$, n = 7, $P < 0.001$; fig. 5B), 10 nmol DL-APV (Friedman test, $\chi^2_8 = 27.3$, n = 7, $P < 0.001$; fig. 5C), and 200 nmol L-NAME (Friedman test, $\chi^2_8 = 36.1$, n = 7, $P < 0.001$; fig. 5D), but not by vehicle (saline) (Friedman test, $\chi^2_8 = 10.1$, n = 7, $P > 0.05$; fig. 5E), at 4 h, but not at days 1, 2, and 3 (data not shown), after incision. The effects of the antagonists or the inhibitor appeared at 20–40 min and lasted at least 120 min after intrathecal injection (figs. 5A–E). The analgesic indices were significantly increased after intrathecal injection of the antagonists or the inhibitor, compared with those after intrathecal injection of vehicle in the neonatally carrageenan-treated rats (Kruskal–Wallis test, $df = 4$, $P < 0.001$; fig. 5F).

Effect of Intrathecal MK-801, DL-APV, and L-NAME on Placing Reflex

Finally, we tested whether the antagonists and the inhibitor at the doses used in the behavioral testing affected the locomotor function. As shown in table 1, except for 30 nmol DL-APV (n = 7), which significantly reduced the placing reflex from 20 to 60 min after injection (Kruskal–Wallis test, $df = 8$, $P < 0.001$), 10 nmol DL-APV (n = 7), 40 nmol MK-801 (n = 7), and 200 nmol L-NAME (n = 7) as well as vehicle (n = 7) did not markedly change the placing reflex during the behavioral observation period. In addition, no significant differences in general behaviors, including spontaneous

Table 1. Effects of NMDA Receptor Antagonist and Nitric Oxide Synthase Inhibitor on Placing Reflex

Group	Paw Tested	Minutes after Intrathecal Injection							
		Pre	20	40	60	80	100	120	180
Saline	Left	6	6	6	6	6	6	6	6
	Right	6	6	6	6	6	6	6	6
MK-801, 40 nmol	Left	6	5	5	6	6	6	6	6
	Right	6	5	5	6	6	6	6	6
DL-AVP, 30 nmol	Left	6	3*	3*	3*	4	5	5	6
	Right	6	3*	3*	4*	4	5	5	6
DL-APV, 10 nmol	Left	6	5	5	6	6	6	6	6
	Right	6	5	6	6	6	6	6	6
L-NAME, 200 nmol	Left	6	6	5	6	6	6	6	6
	Right	6	6	6	6	6	6	6	6

n = 7, three trials.

* $P < 0.05$ *vs.* pretreatment.

DL-APV = DL-2-amino-5-phosphonovaleric acid; L-NAME = L-N^G-nitro-arginine methylester; NMDA = *N*-methyl-D-aspartate.

activity, were observed between the drug-treated and vehicle groups.

Discussion

The current study demonstrates that intraplantar injection of carrageenan into the left hind paw of Sprague-Dawley male rats on postnatal day 1 produces enhanced plantar incision-induced mechanical pain hypersensitivity on the ipsilateral hind paw of adult rats at 4 h after incision. This enhancement was significantly reversed by intrathecal administration of DL-APV, MK-801, and L-NAME. Our findings indicate that neonatal noxious insult could produce a long-term effect on postoperative incisional pain in adults. Spinal cord NMDA receptors and downstream nitric oxide signaling might be involved in the central mechanism that underlies this long-term effect.

Emerging evidence indicates that neonatal noxious insults produce the long-term changes in pain responsivity in adults.[2–5] These changes include basal hypoalgesia and excessive reinflammation-associated hyperalgesia in the adult rats subjected to plantar injection of carrageenan at postnatal days 0–5 (but not at postnatal day 8 or more). Consistent with previous studies,[5,28,29] the current study showed that a single injection of carrageenan into the plantar surface of the left hind paw at postnatal day 1 resulted in significant increases in basal paw withdrawal thresholds in response to mechanical stimuli on both hind paws. We also found excessive incision-associated mechanical pain hypersensitivity on the left hind paw only on the day of surgery (at 4 h) but not on postoperative day 1 or 2 in the neonatally carrageenan-treated group, compared with that in the naive or the neonatally saline-treated group. Moreover, our study showed that plantar incision produced a greater increase in Fos expression, a marker for functional activity of neurons,[20,21] in the ipsilateral spinal dorsal horn of the neonatally carrageenan-treated rats at 2 h after incision, compared with that in the naive or the neonatally saline-treated rats. These findings further support the notion that neonatal local noxious insult could lead to long-term effects on central responses to painful stimuli in adults.

However, the underlying mechanisms by which neonatal pain produces long-term effects on nociceptive systems in adults are still unclear. It has been proposed that the neonatal local nociceptive insult might induce both nociception-enhancing changes and a compensatory boosting of the relevant inhibitory mechanisms.[2,30–32] Under basal conditions, the balance between these two competing mechanisms in the neonatally inflamed animals may favor descending inhibition that masks segmental nociceptive hypersensitivity and results in widespread hypoalgesia. The latter is supported by the observation that long-term hypoalgesia was detected not only in the neonatally carrageenan-injected left hind paw, but also in the intact right hind paw and forepaw.[4,5] Plantar incision in the current study and reinflammation in the previous studies[4,5] at the site of the neonatal inflammatory insult may strengthen the pronociceptive mechanisms, resulting in enhanced pain hypersensitivity on the ipsilateral hind paw and in undetected hypoalgesia on the contralateral hind paw. It is clearly evident that excessive pain hypersensitivity has a local and ipsilateral segmental nature, which is further supported by our observation that the excessive pain hypersensitivity was not reproduced by plantar incision of the right hind paw in the rats with the neonatally inflamed left hind paw. A more recent report revealed significant basal and inflammation-associated aberrations in the expression of multiple genes in the lumbar dorsal horn in the neonatally carrageenan-treated rats.[7] The expression of 12 genes was up-regulated in a bilateral fashion in the dorsal horn at baseline. These genes encode members of several proteins, such as γ-aminobutyric acid synthesis enzymes and receptors, interleukins and their receptors, serotonin, adenosine, neuropeptide Y, cholecystokinin receptors, opioid, and tachykinin peptides.[7] The translational products of the majority of these genes are most likely to enhance inhibitory processing of nociceptive input in the spinal cord. Adult inflammation of the left hind paw induced significant up-regulation in expression of 36 genes in the ipsilateral dorsal horn in the neonatally carrageenan-treated rats, compared with similarly inflamed animals.[7] In particular, eight of the affected genes encode interleukins and their receptors, which are proinflammatory molecules.[32,33] Another eight genes encode glutamate neurotransmission-related proteins, including subunits of NMDA receptors and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, which play mostly pronociceptive

roles in the dorsal horn.[34,35] However, it is still unclear whether the changes in these affected genes are really involved in the molecular mechanisms of long-term alteration in nociception produced by early local noxious insult.

We have noted that the withdrawal mechanical thresholds that were measured in all three groups in our study were lower compared with the previous studies.[12,16,17] The reason for the discrepancy between the previous and current results is unclear but may be related to the different age of the rats that were used in the previous and current studies. Most previous studies used Sprague-Dawley rats with body weight of 300–350 g, which may be aged 2 months or older, whereas we used Sprague-Dawley rats aged younger than 2 months ($\leq$ 57 days; body weight $<$ 250 g). A previous study demonstrated that paw withdraw mechanical thresholds in the younger rats were significantly lower than those in the older rats.[5] In addition, it is possible that the rats from different vendors (especially from different counties) may have distinct behavioral responses to mechanical stimulation, although they are the same species.

The current study demonstrated that activation of spinal cord NMDA receptors and downstream nitric oxide signaling might play a critical role in the central mechanism that underlies excessive incision-associated mechanical pain hypersensitivity in rats subjected to neonatal peripheral inflammation. Intrathecal administration of 40 nmol MK-801, 10 and 30 nmol DL-APV, or 200 nmol L-NAME at 4 h after incision significantly reversed the incision-induced decrease in paw withdrawal threshold in response to mechanical stimuli on the neonatally carrageenan-injected hind paw. Neither the antagonists nor inhibitor at the doses used (except for DL-APV at 30 nmol) produced remarkable effects on locomotor activity, indicating that their effects on excessive incision-associated mechanical pain hypersensitivity in the neonatally inflamed rats may be due to their analgesic action. We also found that intrathecal administration of the antagonists and the inhibitor at the doses used did not affect incision-induced mechanical pain hypersensitivity at 4 h after incision in either the naive group or the neonatally saline-treated groups. This is consistent with previous reports[12,17] that demonstrated that spinal NMDA receptors are not involved in central mechanisms underlying incisional pain in normal rats. How do they produce different effects on incision-induced pain hypersensitivities in the neonatally inflamed group *versus* the naive group or the neonatally saline-injected groups? It is well known that glutamate, the principal excitatory neurotransmitter at the synapses, causes sustained depolarization of dorsal horn neurons, removes the voltage-dependent magnesium block from the NMDA receptor complex, and allows calcium influx. Intracellular calcium increase induces activation of calmodulin-dependent protein kinases, nitric oxide production, and protein phosphorylation.[34,36,37] It is possible that in the naive and the neonatally saline-treated groups, the extent of incisional injury might not be sufficient to activate spinal NMDA receptors, even if it produces the greatest pain hypersensitivity at 4 h after incision.[12] Conversely, in the neonatally carrageenan-treated group, neonatal local inflammatory insult might up-regulate NMDA receptor subunits in adults, which is likely to increase NMDA receptor ligand sensitivity,[38,39] resulting in much easier activation of spinal NMDA receptors evoked by incisional injury at 4 h after incision. This deduction is strongly supported by the aforementioned fact that neonatal inflammatory insult up-regulated the expression of the genes encoding NMDA receptor subunits in the ipsilateral dorsal horn of adult rats during reinflammation.[7] Interestingly, our pilot work showed that MK-801, DL-APV, and L-NAME at the doses used did not affect the incision-induced mechanical pain hypersensitivity in the neonatally carrageenan-treated group at day 1 or 2 after incision. At these two time points, plantar incision–induced mechanical pain hypersensitivity in the neonatally carrageenan-treated group was similar to that in the naive and neonatally saline-treated groups, suggesting that the intensity of noxious inputs from the healing wound at days 1 and 2 after incision might not be enough to activate spinal NMDA receptors, even if spinal NMDA receptor subunits and their ligand sensitivity are increased. Therefore, it is likely that spinal NMDA receptors and downstream nitric oxide signaling might specifically contribute to the central mechanism underlying excessive incision-associated pain hyperalgesia in rats subjected to early local noxious insult.

In conclusion, the current study, for the first time, demonstrated that neonatal pain and inflammation could lead to excessive incision-associated mechanical pain hypersensitivity in adult rats. It has been postulated that the rat central nervous system during the first postnatal week developmentally corresponds to that of prematurely born human infants who usually require placement in the postpartum neonatal intensive care unit for invasive procedures associated with pain and local inflammation.[1,40] The current study suggests that these infants might experience abnormal sensitivity to noxious environmental stimuli, such as surgical incision, in later life. This places special emphasis and urgency on prevention and treatment of pain and inflammation in premature infants. Our current work also suggests that NMDA receptors and downstream nitric oxide signaling might be involved in the central mechanism that underlies this abnormal pain sensitivity. Spinal cord NMDA receptors might be a potential target for treatment of abnormal incisional pain in some patients who are born prematurely.

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