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Guarding Pain and Spontaneous Activity of Nociceptors after Skin *versus* Skin Plus Deep Tissue Incision

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Abstract

Background—Guarding pain after rat plantar incision is similar to pain at rest in postoperative patients. Spontaneous activity (SA) in nociceptive pathways quite likely transmits such ongoing pain. This study examined the extent of tissue injury by incision on pain behaviors and nociceptor SA.

Methods—Rat pain behaviors were measured after a sham procedure, skin incision, or skin plus deep tissue incision. Separate groups of rats underwent *in vivo* single-fiber recording 1 day after a sham procedure, skin, or skin plus deep tissue incision or 7 days after skin plus deep tissue incision.

Results—Compared with the control procedure, skin incision induced moderate guarding on the day of incision only, whereas skin plus deep tissue incision caused guarding for 5 days. Mechanical and heat hyperalgesia were similar in both incised groups, except that mechanical hyperalgesia lasted longer after skin plus deep tissue incision. On Postoperative Day 1, skin incision (18.2%) produced a similar prevalence of SA in nociceptors as in controls (13.0%), whereas skin plus deep tissue incision generated a greater prevalence of SA (61.0%); SA rate also tended to be greater (6.1 *vs.* 10.0 imp/s) after skin plus deep tissue incision. Seven days after skin plus deep tissue incision, the SA prevalence was similar (13.6%) as in controls.

Conclusions—These data demonstrated that incised deep tissue rather than skin had a central role in the genesis of guarding behavior and nociceptor SA. Understanding the responses of deep tissue to incision and the mechanisms for deep tissue pain will improve postoperative pain management.

IN pain research, the pathophysiology of sensitization that produces allodynia and hyperalgesia in postinjury states has generated considerable interest. Ongoing, unprovoked pain after injury is less well studied. In patients with postoperative pain, this is measured as pain at rest, which should be transmitted by spontaneous activity (SA) from nociceptors. Generally, most nociceptors have no SA, and the generation of SA in postinjury states is perhaps the most powerful form of sensitization in nociceptive pathways. Several investigators have used needles or scalpels to injure the receptive field (RF) of skin nociceptors but did not generate SA, even though marked ongoing pain would be expected immediately after acute injury.[1–3]

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To better understand postoperative pain, we have examined a variety of pain-related behaviors and explored the underlying mechanisms for postoperative pain using an incisional pain model.[4] Unprovoked pain-related behavior, guarding, after rat plantar incision has been described and suggested to be a correlate to the pain at rest in patients.[4–6] For example, pain at rest after surgery is greatest in the recovery room[7] and on the first few days after surgery,[8–10] and guarding pain is greatest immediately after plantar incision and gradually diminishes over several days.[4,11] It is expected that an incision and acute tissue damage should generate the greatest SA immediately after injury, and the SA should gradually diminish over hours or days.

What We Already Know about This Topic

❖ Mechanisms causing pain at rest after surgery are unknown

What This Article Tells Us That Is New

❖ In rats, skin incision had little effect on guarding behavior, a measure of pain at rest, or spontaneous nociceptor activity

❖ Incision of skin and deeper tissue increased guarding behavior and spontaneous activity, suggesting that pain at rest after surgery reflects sensory nerve response to deep tissue injury

However, in previous studies, SA was not evident in mechanosensitive afferents immediately after plantar incision,[3] whereas significant SA was present 1 day after incision.[12] We first suggested that nociceptors, which developed SA after incision, were mechanically insensitive afferents or chemosensitive afferents.[13–15] These afferents are not easily identified by using a mechanical search stimulus in normal tissue before incision[3] but can be sensitized and distinguished 1 day after tissue injury.[12] However, when we applied an electrical stimulus to skin to recruit both mechanosensitive and mechanoinsensitive afferents, SA was still not evident immediately after plantar incision, indicating that mechanoinsensitive skin afferents were not the origin of SA.

In this study, we hypothesized that incision of skin was not sufficient to cause persistent activation of nociceptors, but incision into deep tissue produced the majority of SA in nociceptors and guarding behavior after plantar incision. Our recent studies showed that skin incision induced modest SA in dorsal horn neurons (DHNs), whereas much greater SA was present after skin plus deep tissue incision.[16,17] In addition, this high SA in DHNs after skin plus deep tissue incision could be inhibited by intraplantar bupivacaine injection, indicating a causal role of primary afferent input.[16,17]

We examined the entire time course of pain behaviors of rats with a sham incision procedure, skin incision, or skin plus deep tissue incision. SA and responses to mechanical and heat stimuli of nociceptors were recorded *in vivo* from separate groups of rats 1 day after sham procedure, skin incision, or skin plus deep tissue incision or 7 days after skin plus deep tissue incision. Pain behaviors were also measured on the same rats that underwent nerve recordings.

Materials and Methods

General

All experiments were reviewed and approved by the University of Iowa Animal Care and Use Committee (Iowa City, Iowa). Rats were treated in accordance with the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals as issued by the

International Association for the Study of Pain.[18] Adult male Sprague-Dawley rats (280–320g, Harlan, Indianapolis, IN) were used for behavioral and electro-physiological studies. Rats were housed on a 12-h light–dark schedule; food and water were available *ad libitum*.

Surgical Incisions

This study used two types of incisions: skin incision that incised only the epidermis and dermis of the skin and skin plus deep tissue incision that incised skin and underlying fascia and the plantar flexor digitorum brevis muscle. Detailed methods for performing these incisions were described in our previous article.[16] Briefly, anesthesia was induced by placing the animal in a sealed plastic box filled with 5% isoflurane mixed with air. During surgery, anesthesia was maintained with 1.5–2% isoflurane delivered through a nose cone to the animal. The left hind paw of the rat was sterilized with 10% povidone-iodine. For the skin incision group, a blade handle was designed to hold a #11 blade (Feather Co., Osaka, Japan) and control the incision depth to 0.4 mm, as described previously.[16] Beginning 0.5 cm from the proximal edge of the heel, a 1-cm longitudinal incision was made. The incised skin was then closed with three subcutaneous mattress sutures with 6-0 nylon on a P-1 needle (Ethicon, Somerville, NJ) while being visualized under a binocular dissecting microscope. The wound was then covered with antibiotic ointment.

The skin plus deep tissue incision was made similar to that described previously.[4] At the same plantar site as for the skin incision, a 1-cm longitudinal incision was made through the skin and underlying fascia and the plantar flexor digitorum brevis muscle with a #11 surgical blade. Blunt curved forceps were then inserted through the incision into the muscle to further divide and retract the muscle. The muscle origin and insertion remained intact. This method was similar to that described previously[4] except that the muscle was not elevated at its dorsal surface. The wound was then closed with three subcutaneous mattress sutures as for the skin incision.

Rats in the control group received a sham surgical procedure. Rats were anesthetized with isoflurane, the hind paw was prepared using a sterile procedure, and topical antibiotics were applied, but no incision was made. For the postoperative day (POD) 7 rats, sutures were removed on POD2. The person performing the behavioral and electrophysiological studies was blinded to the type of incision on POD1 but could not be blinded to sham or POD7 incision.

Pain Behaviors

The time course for changes in pain behaviors after the different types of incisions was examined in three groups of rats: sham control, skin incision, and skin plus deep tissue incision. Each group contained eight rats that underwent tests of guarding pain behavior, withdrawal threshold to mechanical stimuli, and withdrawal latency to radiant heat in this sequence. Detailed methods for these behavioral tests were described previously.[4,16] Briefly, rats were first acclimated in the behavioral testing room for 3 days. Then a baseline test was performed 2 days and 1 day before incision. After incision, pain behaviors were continuously measured once daily from 4h to7 days after incision. The person performing the behavioral test was blinded to incision types: skin incision *versus* skin plus deep tissue incision.

For guarding behavior measurement, rats were placed individually on a small plastic mesh floor (grid 8 × 8 mm) covered with a clear plastic cage top (21 × 27 × 15 cm). Both incised and nonincised hind paws were closely observed during a 1-min period repeated every 5 min for 1 h. According to the hind paw position during the majority of the 1-min scoring period, a score of 0, 1, or 2 was given. Zero was scored when the incised area was touching the

mesh, and the area was blanched or distorted by the mesh; 1 was scored when the incised area touched the mesh without blanching or distortion; 2 for the position when the incised area was completely off of the mesh. For each hind paw, a sum score was obtained by adding the 12 scores during the 1-h testing period. The guarding score was then obtained by subtracting the score of the incised hind paw from that of the nonincised hind paw.

For mechanical withdrawal threshold measurement, rats were placed on a plastic mesh floor with 12 × 12 mm openings. Calibrated monofilaments with bending forces of 13, 19, 59, 73, 98, 112, 139, and 228 mN were used (Stoelting, Wood Dale, IL). The filament was carefully applied from underneath the mesh to an area adjacent to the incision or to a corresponding area in the nonincised hind paw. Starting with 13 mN, each filament was applied once until a withdrawal response was evoked. If the force of 228 mN was reached and there was still no withdrawal response, then 673 mN, the bending force of the next filament, was recorded as the threshold. This test was performed for three times with a 5-min interval between two tests. The lowest force that elicited a response from the three tests was defined as the mechanical withdrawal threshold.

For heat withdrawal latency measurement,[19] rats were placed individually on a glass floor covered with a clear plastic cage. Radiant heat from a 50-W projector lamp was applied to the incision or the corresponding area in the nonincised hind paw from underneath the glass floor. The latency to evoke withdrawal was determined with a cutoff value of 20 s. Each rat was tested three times with an interval of 10 min. The average of the three trials was recorded as the heat withdrawal latency.

Electrophysiological Studies

General—Separate groups of rats were subjected to *in vivo* single-fiber recording: sham control (six rats), skin incision (seven rats), skin plus deep tissue incision (POD1, seven rats), and skin plus deep tissue incision (POD7, seven rats). Incisions were performed as described in the surgical incision methods. Before the electrophysiological experiment, the same rats underwent brief behavioral tests. The methods of behavioral measurement were the same as described earlier except that the testing period for guarding behavior was decreased from 60 to 30 min and only one mechanical test and one heat test (rather than three) were performed to lessen the time to begin electrophysiological experiments. For the sham control group, the skin incision group and the skin plus deep tissue incision (POD1) group, behaviors were measured 1 day after incision or sham surgery. For the skin plus deep tissue incision (POD7) group, behaviors were measured 7 days after incision.

Preparation—The procedures for *in vivo* fiber recording were described in detail previously.[3,12] Briefly, rats were initially anesthetized with an intraperitoneal injection of pentobarbital sodium (Nembutal; 50 mg/kg). Supplementary doses of pentobarbital sodium (20 mg/kg) were given approximately every hour during the recording period. The hair on the neck and left leg was clipped. The common carotid artery was cannulated for measuring the blood pressure. A tracheotomy was performed, and the rat was ventilated (Harvard Apparatus, Inc., South Natick, MA). During the experiment, the mean arterial blood pressure was above 90 mmHg, or the experiments were terminated. The body temperature was maintained at approximately 37°C with a servo-controlled electric heating lamp and an underbody heating pad. At the popliteal fossa, a 2-cm longitudinal incision in skin was made in the left leg toward the heel. After the underlying connective tissue was divided bluntly with curved forceps, the tibial nerve was isolated and transected proximally. A pool for warm mineral oil was then made over the exposed underlying tissue by suturing the incised skin to a metal ring (1.5 cm ID), which was attached to a micromanipulator. The left hind paw of the rat was extended and placed on a silicone stage with the plantar aspect exposed

for further testing. The proximal cut end of the nerve was placed and desheathed on a mirrored stage immersed in the mineral oil pool. Fine filaments of the tibial nerve were repeatedly teased from the cut end and placed on a platinum electrode until single-unit activity could be discriminated and recorded. A reference electrode was pinned onto the adjacent muscle tissue. Nerve activity was amplified (DAM50, Harvard Apparatus, Holliston, MA), filtered, and displayed on a digital oscilloscope. All data were recorded and stored into a personal computer with a data acquisition system (1401 Plus Laboratory Interface and spike2 program, Cambridge Electronic Design, Cambridge, United Kingdom).

Recording Protocol—Gentle tapping with the experimenter's index finger and a suprathreshold 228 mN von Frey filament to the plantar hind paw were used as search stimuli. Fibers were accepted for further study if their RF included at least part of the incision or the corresponding nonincised site, and the signal to noise amplitude was at least 2:1 *versus* other activities.

Once an afferent fiber that fulfilled the search criteria was identified, ongoing SA of the fiber was first recorded for 10 min. The activity during the latter 5-min period was averaged and analyzed. An afferent fiber with a mean activity of at least 0.1 imp/s (a minimum of 30 impulses during the 5-min period) was considered spontaneously active.

After the recording of SA, the mechanical RF was evaluated using a von Frey filament with a bending force of 228 mN and then drawn on a schematic of the plantar hind paw accordingly. The schematic was then scanned. The relative size of the RF was measured with the National Institutes of Health Image J software (National Institutes of Health, Bethesda, MD).

Next, afferent responses to mechanical stimulation were tested at the most sensitive site of the mechanical RF. Calibrated von Frey filaments with bending forces of 5, 8, 13, 29, 41, 60, 67, 93, 133, and 228 mN were applied to the RF in an ascending order. Each filament was applied once for approximately 3 s. The interstimulus interval was 5–10 s. Using a 1-s bin width, the peak response rate (in impulse per second) of the fiber was defined as the greatest rate during application of the stimulus. To obtain a stimulus response function, the peak response rate of the fiber during application of von Frey filaments was averaged and plotted *versus* the force of each filament. The mechanical response threshold of each afferent fiber was defined as the lowest force that caused either activation of the fiber if no SA was present or an increase in fiber discharge rate by at least two SDs above mean SA before the application of filaments.[12] The next strength filament must also have excited the fiber.

To examine the thermal responses of afferent fibers and exclude cool fibers from nociceptive afferents analyses, after the mechanical stimuli, both heat and cool stimuli were applied to the RF through a feedback-controlled Peltier device (Yale Instrumentation, New Haven, CT). The thermal probe had a contact area of 1 cm^2 and was connected to a micromanipulator. The thermal probe was positioned onto the mechanical RF. The RF was first exposed to a baseline temperature (32°C) for 1 min. After the adaptation temperature, a heat ramp from 32° to 45°C was delivered in 10 s. To avoid potential tissue damage, no test was performed at a temperature greater than 45°C. The temperature remained at the peak (45°C) for 1 s and then decreased to 32°C in 10 s. After completing the heat test, the thermal probe remained in the same position, and the temperature of 32°C was maintained. One minute after the heating, a cooling ramp from 32° to 22°C was delivered to the RF over 10 s. The temperature remained at 22°C for 1 s and then increased to 32°C in 10 s. The discharge of the afferent fiber was continuously recorded during the thermal experiment. The fiber was activated by heat or cooling if discharge was evoked when there was no SA present before

the stimulation, or the discharge rate during the stimulation was increased by at least two SDs above the baseline SA (10 s, 1-s bin) when SA was present before testing. The fiber was inhibited by heat or cooling if the discharge rate was decreased by at least two SDs below the baseline SA (10 s, 1-s bin). A cool fiber was identified as a spontaneously active fiber, which was activated by cooling and inhibited by heat. These nonnociceptive cool fibers were excluded from the final analysis.

At the end of each recording, the fiber conduction velocity (CV) was measured. Two needle electrodes were inserted transcutaneously (approximately 1.0–3.0 mm deep from the plantar surface of the skin) 5 mm proximal and distal to the incision; electrical stimulation with increasing current and duration (0.5 to 50 mA and 0.05- to 1-ms pulses, 0.2–1.0 Hz) was delivered to evoke action potentials from the fiber as described previously.[12] For some afferent fibers, action potentials could not be elicited even with strong electrical stimulation. In such cases, the two needle electrodes were placed closer to the mechanical RF proximally and distally. With the closer position of the stimulating electrodes, most afferent CVs could be measured. Once a needle electrode was inserted within the incision area, no subsequent fiber was recorded from that region. The CV was calculated by dividing the distance between the RF and the recording electrode by the latency of the evoked action potential. If the CV could not be measured, the fiber was not included in the analysis.

Mechanosensitive Aδ- and C-nociceptors were studied. An afferent fiber was classified as an Aδ-fiber if the CV was greater than 2.5 and equal or less than 25 m/s, as a C-fiber if the CV was equal or less than 2.5 m/s.[20] Mechanosensitive nociceptors were determined according to their graded responses to innocuous and noxious range of mechanical stimuli. Rapidly adapting fibers were not studied.

Statistical Analysis

For continuous data, normal distribution of values was determined by the Kolmogorov-Smirnov test. Guarding pain behavior and withdrawal latency to heat of rats were compared among groups by two-way ANOVA followed by separate one-way ANOVAs with Tukey *post hoc* test at each time point. For withdrawal threshold to mechanical stimulation of rats, Friedman test two-way ANOVA and Kruskal-Wallis test with Dunn *post hoc* test at each time point for among groups were used.

One way ANOVA with Tukey *post hoc* test was used to analyze CV of units, rate of SA, and RF area among groups. Chi-square test with Bonferroni correction for multiple tests was performed to analyze the prevalence of SA and cumulative percentage of mechanoresponsive fibers at each force. Kruskal-Wallis test with Dunn *post hoc* test was used for comparing the mechanical response threshold of units among groups. Two-way ANOVA followed by separate oneway ANOVAs with Tukey *post hoc* test was used to analyze mechanical stimulus response function at each force range among the four groups. All results are expressed as mean ± SEM or median with range. A P value less than 0.05 was considered statistically significant. All tests were performed with GraphPad Prism software (GraphPad, San Diego, CA).

Results

Behavioral Studies

Skin incision induced guarding to 5.9 ± 2.2 and this was greater than that of the sham control (0.8 ± 0.7) at 2 h after incision ($P < 0.05$, fig. 1A). Compared with sham control, skin plus deep tissue incision caused greater guarding pain from 2 h (19.9 ± 1.1, $P < 0.001$) to 5 days (5.1 ± 1.1, $P < 0.01$) after incision. Skin plus deep tissue incision also induced greater guarding pain than skin incision from 2 h ($P < 0.001$) to 5 days ($P < 0.05$).

For mechanical responses, skin incision induced lower mechanical withdrawal threshold than sham control from 2 h (median: 59 *vs.* 673 mN, $P < 0.01$) to 3 days (183.5 *vs.* 673 mN, $P < 0.05$) after incision (fig. 1B). Compared with the sham group, the skin plus deep tissue incision group had lower withdrawal thresholds from 2 h (59 *vs.* 673 mN, $P < 0.001$) to 5 days after incision (228 *vs.* 673 mN, $P < 0.01$). When the skin plus deep tissue incision group was compared with the skin incision group, no difference was present from 2 h to 3 days after incision; differences occurred only at 4 days (125.5 *vs.* 673 mN, $P < 0.01$) and 5 days (228 *vs.* 673 mN, $P < 0.05$) after incision.

For heat responses, the skin incision group had lower withdrawal latencies than that of the sham control group from 2 h (3.6 ± 0.4 *vs.* 11.5 ± 0.5 s, $P < 0.001$) to 4 days after incision (8.6 ± 0.5 *vs.* 12.2 ± 0.2 s, $P < 0.001$, fig. 1C); the skin plus deep tissue incision group had lower withdrawal latencies than that of the sham group from 2 h (2.8 ± 0.2 *vs.* 11.5 ± 0.5 s, $P < 0.001$) to 5 days after incision (7.5 ± 0.6 *vs.* 11.3 ± 0.5 s, $P < 0.001$). No difference in heat withdrawal latency was evident between the skin incision group and the skin plus deep tissue incision group throughout the 7-day testing period.

For the rats that also underwent nerve recordings, the abbreviated pain testing on POD1 produced similar results to the full 1-h tests. No difference in guarding pain was present between sham procedure (0.7 ± 0.7) and skin incision (1.3 ± 1.5); the skin plus deep tissue group had greater guarding pain (7.8 ± 0.6) than the sham group ($P < 0.001$, fig. 2A). Seven days after skin plus deep tissue incision, guarding pain (0.2 ± 1.0) had resolved; values did not differ from the sham control group. The median mechanical withdrawal threshold in the sham control group (673 mN) was higher than that in the skin incision (98 mN, $P < 0.05$) and the skin plus deep tissue incision (POD1) groups (73 mN, $P < 0.01$) and similar to that in the skin plus deep tissue incision (POD7) group (673 mN, fig. 2B). The heat withdrawal latency in the sham control group (12.3 ± 0.7 s) was also higher than that in the skin incision (5.0 ± 0.5 s, $P < 0.001$) and the skin plus deep tissue incision (POD1) groups (4.5 ± 0.2 s, $P < 0.001$) and similar to that in skin plus deep tissue (POD7) group (14.5 ± 1.5 s, fig. 2C).

Neurophysiologic Studies

A total of 90 mechanosensitive nociceptors from 27 rats were recorded and included in the final analysis. There were 23 fibers (4 Aδ- and 19 C-fibers) in the sham control group, 22 fibers (3 Aδ- and 19 C-fibers) in the skin incision group, 23 fibers (5 Aδ- and 18 C-fibers) in the skin plus deep tissue incision (POD1) group, and 22 fibers (7 Aδ- and 15 C-fibers) in the skin plus deep tissue incision (POD7) group. All fibers had a mechanical RF, which included at least part of the incision or the corresponding site in the unincised hind paw.

Examples of CV measurement of an Aδ-fiber and C-fiber were shown in figures 3A and B, respectively. The average CV of Aδ-fibers was 4.2 ± 0.9, 3.6 ± 0.5, 12.5 ± 3.8, and 7.8 ± 1.6 m/s in the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively (fig. 3C). The average CV of C-fibers was 0.9 ± 0.2, 1.2 ± 0.2, 1.2 ± 0.2, and 0.9 ± 0.1 m/s in the four groups, respectively (fig. 3D). No difference in CV was found among groups for either Aδ- or C-fibers.

Spontaneous Activity

SA was present in both Aδ- and C-nociceptors. Examples of SA of a nociceptor from skin plus deep tissue incision (POD1) group is shown in figure 4A. Overall, the percentage of nociceptors with SA was 13.0% (25% Aδ- and 10.5% C-nociceptors), 18.2% (33.3% Aδ- and 15.8% C-nociceptors), 61.0% (60.0% Aδ- and 61.1% C-nociceptors), and 13.6% (0% Aδ- and 20.0% C-nociceptors) in the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively (fig. 4B).

The sham group had a similar prevalence of SA of nociceptors as the skin incision group and the skin plus deep tissue incision (POD7) group; the prevalence of SA in the skin plus deep tissue incision (POD1) group was greater than that in the sham group ($P < 0.01$). The skin plus deep tissue incision (POD1) group also had more nociceptors with SA than the skin incision group ($P < 0.05$) and the skin plus deep tissue incision (POD7) group ($P < 0.01$).

The mean rate of SA was 6.1 ± 5.9, 5.6 ± 3.2, 10.0 ± 3.2, and 0.3 ± 0.1 imp/s in the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively (fig. 4C). Although the rate of SA in the skin plus deep tissue incision (POD1) group was the greatest, no significant differences were found among these groups.

Responses to Mechanical Stimulation

Examples of mechanical responses of nociceptors from sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups are shown in figures 5A–D, respectively. The median mechanical threshold for Aδ-nociceptors was 61 mN (range, 5–93 mN), 8 mN (5– 8 mN), 8 mN (5– 41 mN), and 60 mN (8 –228 mN) in the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively (fig. 6A). No difference in threshold was present among the groups perhaps because of the small number of fibers in each group. The median threshold for C-nociceptors was 29 mN (range, 5–67 mN), 13 mN (5–67 mN), 13 mN (5–67 mN), and 67 mN (5–228 mN) in the 4 groups, respectively (fig. 6B). The sham control group tended to have a higher threshold compared with the skin incision and skin plus deep tissue incision (POD1) group, but these differences were not significant. Both the skin incision group ($P < 0.01$) and the skin plus deep tissue incision (POD1) group ($P < 0.01$) had lower thresholds than the skin plus deep tissue incision (POD7) group.

Both Aδ- and C-nociceptors showed increased responses to greater filament forces (two-way ANOVA, $P < 0.001$, figs. 6C–F). Because skin incision was sufficient for mechanical hyperalgesia after incision and most SA seemed to be generated by deep tissue incision, the coding of mechanical hyperalgesia in nociceptors should be present in those without SA. This was true for DHNs.[16,17] For Aδ-fibers with and without SA, the number in each group was too small for statistical analysis, and marked differences were not evident. This was also the case for the C-fibers with SA. For C-fibers without SA, no difference in mechanical responses was present among groups. Examples of RF areas of Aδ-fibers and C-fibers were shown in figures 7A and B, respectively. For Aδ-nociceptors, the normalized RF size was 100% ± 18%, 202% ± 45%, 183% ± 20%, and 132% ± 32% in the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively (fig. 7C). No difference in RF size of Aδ-nociceptors was found among groups. For C-fibers, the normalized RF size was 100% ± 11%, 199% ± 21%, 375% ± 49%, and 112% ± 21% in the four groups, respectively (fig. 7D). The skin plus deep tissue incision (POD1) group had greater RF size than the sham control group ($P < 0.001$), the skin incision group ($P < 0.01$), and the skin plus deep tissue incision (POD7) group ($P < 0.001$). Although skin incision increased RF size, it was not significant *versus* sham control in this study.

Responses to Thermal Stimulation

During the experiment, all fibers were tested for responses to heat and cooling. There were 4.3% (1 of 23), 4.5% (1 of 22), 8.8% (2 of 23), and 9.1% (2 of 22) of nociceptors responsive to 45°C heat in the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively. The heat threshold was 44.3°,

40.6°, 38.9° and 43.5°, and 45.0° and 44.0°C in the four groups, respectively. The number in each group was too small for statistical analysis.

There were seven units defined as cool fibers: 1, 3, 1, and 2 units from the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively. These cool fibers all had SA, which was increased by cooling and decreased by heat. These nonnociceptive SA fibers were excluded from the final analysis.

Discussion

This study examined the entire time course of pain behaviors after skin *versus* skin plus deep tissue incision. Most importantly, the prevalence of SA in nociceptors on POD1 was greater than that of the sham control group only when the deep tissue was incised; the prevalence of SA was the same as the sham control 1 day after skin incision. The marked SA of nociceptors occurred when guarding was apparent on POD1; as guarding pain behavior resolved on POD7, the prevalence of SA of nociceptors was the same as the sham control group. Skin incision did not produce guarding pain on POD1 and thereafter, whereas skin plus deep tissue incision caused guarding. The data revealed that incision of skin alone was sufficient to induce heat hyperalgesia; incision of deep tissue did not further enhance heat responses. Skin incision was also sufficient to reduce mechanical withdrawal threshold early after incision; the incised deep tissue contributed to the mechanical hyperalgesia at later times. Cool fibers did not contribute to the high prevalence of SA after incision.

Guarding Pain and SA in Nociceptive Pathways

In previous studies, guarding was inhibited by very low doses (0.03–0.1 mg/kg) of parental morphine administration,[11] local anesthetic infiltration,[21] and nerve growth factor sequestration.[22,23] Both local infiltration and perineural application of capsaicin also attenuated guarding after incision,[24] which had an analgesic effect in patients after inguinal hernia repair.[25] These pharmacological data together with the similarities in the time course of postoperative pain and guarding suggest that guarding may be a clinically relevant behavior to understand pain at rest after surgery.

Guarding pain also correlates with SA in DHNs.[16,17] Marked SA was present on POD1, whereas on POD7 when guarding pain was not evident, the SA was also the same as that in sham-operated rats.[16] In support of this concept of SA causing guarding pain, intrathecal non-*N*-methyl-$_D$-aspartate receptor antagonists inhibited guarding pain and SA in DHNs after incision,[26] whereas blockade of spinal *N*-methyl-$_D$-aspartate receptors did not affect either guarding pain or DHN SA produced by incision.[6,26]

Incision of Skin versus Deep Tissue and SA in Nociceptors

Most nociceptors do not have SA in the normal state.[3,12,27,28] Several other investigators have used mechanical damage or skin cuts to attempt to activate and sensitize cutaneous nociceptors. In these earlier studies, persistent activation of nociceptors was not reported. For example, SA in feline cutaneous A-nociceptors[2] and C-nociceptors[1] was not induced immediately after needle penetrations into the skin RF using a sharp (needle) point *in vivo*. Even after repeated penetrations of the RF in skin, only a few after discharges were occasionally produced.[1] Afferent SA was not reported immediately after cuts (~1 mm deep) were made with a blade in the mechanical RF of cutaneous C-nociceptors of monkey *in vivo*,[29] and in our own *in vivo* studies, after determining the mechanical RF of cutaneous nociceptors, incision was made through the RF, and SA was not generated afterward[3] despite the fact that in behavioral studies, guarding pain was greatest immediately after

incision.[4] Thus, incisions do not seem to generate SA of cutaneous nociceptors (fig. 8). In the *in vitro* studies using a skin-nerve preparation, increased SA was noted in mechanosensitive nociceptors 1 day after plantar incision.[30,31] However, the SA in cutaneous afferents after incision was low (0.6 imp/s for C fibers). Direct comparisons between these data and the current results have limitations due to different experimental conditions, *in vitro versus in vivo*.

In this study, we showed that these afferents with SA after incision quite likely innervate deep tissue (fig. 8). Data from other studies using nerve transection were consistent with our findings. SA was more prevalent in cut medial gastrocnemius nerve afferents than in transected cutaneous (saphenous and sural nerve) afferents in both rat (68.5% *vs.* 3.5–8.1%)[32,33] and cat (37.5% *vs.* 0%).[34] After nerve transection, SA was also evident in the dorsal root ganglion neurons supplying the muscle (~20%) but not in those supplying the skin (0%).[35] In those studies, however, SA was predominant in myelinated muscle afferents. In this study and in Pogatzki *et al.*,[12] both myelinated and unmyelinated fibers possessed SA. An injury in the nerve endings in our model rather than in the axon, as used by others, may in part account for these differences.

Mechanical Responses

The mechanical withdrawal thresholds were reduced to a similar extent by skin incision and skin plus deep tissue incision immediately and from Day 1 to Day 3 after incision. The reduced withdrawal threshold after both types of incisions was comparable with the mechanical pain threshold of humans after skin incision.[36,37] These data suggest that incision in skin alone is sufficient to induce mechanical hyperalgesia as measured by the withdrawal threshold. In agreement, primary mechanical hyperalgesia was also evident for several days after incision in the rat hairy skin.[38,39] When an incision of skin and fascia was compared with an incision of skin, fascia, and muscle, similar primary mechanical hyperalgesia was also produced, indicating a minor role for fascia in mechanical hyperalgesia after plantar incision.[4,19] In this study, compared with skin incision, however, skin plus deep tissue incision caused mechanical hyperalgesia for a longer period (3 *vs.* 5 days).

Previous studies on DHNs noted a difference in mechanical responses between neurons with SA and neurons without SA.[16,17] Those without SA seemed to transmit the responses and elicit the reduced withdrawal threshold after skin incision or after skin plus deep tissue incision. In this study, when stimulus-response relations were compared among groups, differences were not present or could not be tested. This was in part due to the small numbers of nociceptors in some groups.

In this study, the average nociceptor RF size was increased 1 day after skin plus deep tissue incision. Nociceptor RF expansion has also been reported after other injuries and chemical stimuli.[40–42] Because our data suggested that some of these afferents identified by SA quite likely innervated deep tissue, this large size of the mechanical RF might reflect that nociceptors from deep tissue had a large projecting RF area onto skin. This is a limitation when using monofilament testing to examine the RF. The RF of group IV muscle nociceptors in humans, estimated by applying mechanical stimulation on the overlying skin, can have an average size of 3.4 cm^2,[43] which is quite large compared with human cutaneous RFs of C-nociceptors (0.56–1.06 cm^2).[44,45] As we have proposed previously,[3,12] RF expansion could lead to more fibers being recruited by a single mechanical stimulus. Thus, it could produce spatial summation of inputs to DHNs and contribute to mechanical hyperalgesia.

Heat Responses

Both skin incision and skin plus deep tissue incision produced similar changes in heat withdrawal latency, indicating that incision in skin alone is sufficient for development of heat hyperalgesia. Based on the limited number of units, the heat response threshold of nociceptors was reduced to a similar extent by skin incision and skin plus deep tissue incision. In agreement, responses of cutaneous C polymodal nociceptors to heat were significantly increased after skin incisions were made in their RF.[29]

In this study, a small percentage of mechanosensitive nociceptors (4.3–9.1% in the four groups) responded to heat stimulation. In studies by others, heat stimuli (5°C/step, from 32° to 52°C, each step for 20 s) were applied to the rat plantar hind paw; 18.2% Aδ- and 77.2% C-mechanosensitive nociceptors responded to heat.[20] Differences in heat stimulation protocols may account for the disparities between our study and the results of Leem *et al.* In our study, a continuous heat ramp from 32° to 45°C in 10 s was used. One other *in vivo* study agrees with our prevalence of heat-responsive fibers. A comparable incidence of mechano-heat-sensitive nociceptors was reported when hot water (50–65°C) was applied to the cutaneous RF during recordings from dorsal horn ganglion neurons: 2.6% Aδ- and 10.9% C-mechanosensitive nociceptors responded to the heat.[46]

Clinical Implications

Our studies on the rat incisional model lead to the recognition that deep tissue rather than skin has a major impact on ongoing resting pain after surgery. Data from human studies are consistent with this concept. First, a 4-mm-long skin incision in the volar forearm induced a very low level of ongoing pain, which persisted for only 30 min.[36,37] Second, comparisons of surgical approaches for unilateral total hip arthroplasty provide direct evidence. When two approaches with the same length of skin incision (20 cm) and different amount of deep muscle tissue injury were compared, the approach that incised more deep tissue induced greater pain at rest and morphine use.[47] In contrast, when two approaches with different lengths of skin incision (9 *vs.* 16 cm) and the same amount of deep muscle tissue injury were compared, no difference was found in both pain at rest and morphine use.[48]

Conclusion

This study demonstrates that deep tissue rather than skin is critical for the development of guarding after plantar incision and SA of nociceptors (fig. 8). Nociceptor SA was strongly associated with guarding behavior after incision. In contrast, skin incision alone is sufficient to induce primary mechanical and heat hyperalgesia. This study suggests that greater attention should be paid to incised deep tissue and to mechanisms for deep tissue pain to improve postoperative pain management.

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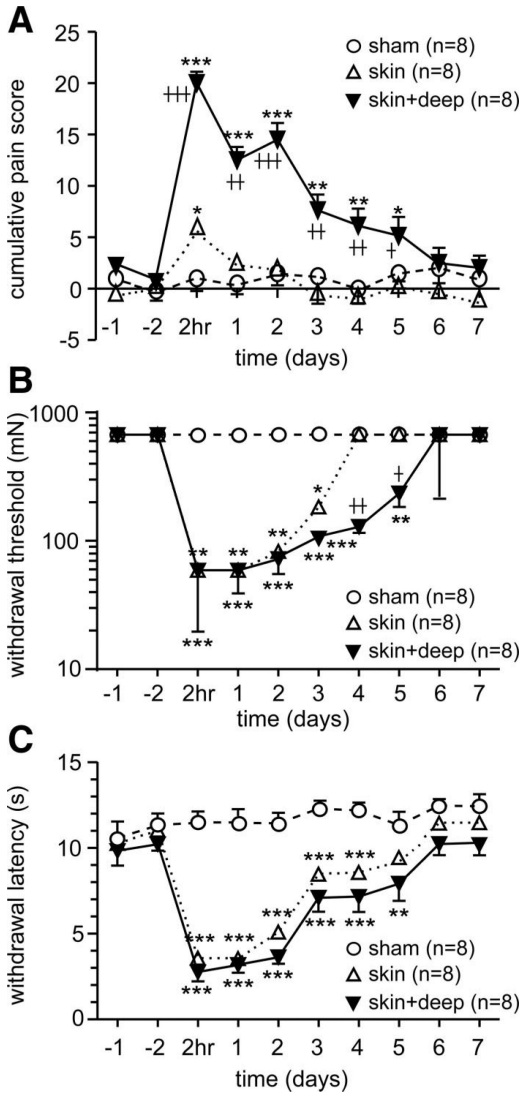

Fig. 1.

Pain behaviors of rats with sham procedure, skin, or skin plus deep tissue incision. (*A*) Guarding pain behavior measured during a 60-min test period. The results are presented as mean and SEM. Two-way ANOVA (interaction factor: $F_{18,210} = 11.5$, $P < 0.0001$) followed by separate one-way ANOVAs with Tukey *post hoc* test for comparing the mean cumulative pain score at each time point among groups (2 h: $F_{2,21} = 50.0$, $P < 0.0001$; 1 day: $F_{2,21} = 18.9$, $P < 0.0001$; 2 days: $F_{2,21} = 36.3$, $P < 0.0001$; 3 days: $F_{2,21} = 10.6$, $P = 0.0006$; 4 days: $F_{2,21} = 8.3$, $P = 0.0022$; 5 days: $F_{2,21} = 6.8$, $P = 0.0051$; 6 days: $F_{2,21} = 2.6$, $P = 0.1016$; 7 days: $F_{2,21} = 2.4$, $P = 0.1163$). (*B*) Withdrawal threshold to von Frey filament application. The results are presented as median with interquartile range. Friedman test (Fr = 15.2, $P < 0.0001$) followed by Kruskal-Wallis test with Dunn *post hoc* test for between-group comparisons at each time point (2 h: $KW_{2,21}$ statistic = 19.1, $P < 0.0001$; 1 day: $KW_{2,21}$ statistic = 18.3, $P < 0.0001$; 2 days: $KW_{2,21}$ statistic = 16.7, $P = 0.0002$; 3 days: $KW_{2,21}$ statistic = 21.0, $P < 0.0001$; 4 days: $KW_{2,21}$ statistic = 18.2, $P = 0.0001$; 5 days: $KW_{2,21}$ statistic = 12.0, $P = 0.0024$; 6 days: $KW_{2,21}$ statistic = 6.5, $P = 0.038$; 7 days: $KW_{2,21}$ statistic = 2.0, $P = 0.37$). (*C*) Withdrawal latency to heat stimulation. The results are presented as mean and SEM. Two-way ANOVA (interaction factor: $F_{18,210} = 11.5$, $P < 0.0001$) followed by separate one-way ANOVA with Tukey *post hoc* test for comparing the

mean withdrawal latency at each time point among groups (2 h: $F_{2,21} = 170.3$, $P < 0.0001$; 1 day: $F_{2,21} = 152.2$, $P < 0.0001$; 2 days: $F_{2,21} = 100.6$, $P < 0.0001$; 3 days: $F_{2,21} = 31.6$, $P < 0.0001$; 4 days: $F_{2,21} = 33.8$, $P < 0.0001$; 5 days: $F_{2,21} = 8.7$, $P = 0.0018$; 6 days: $F_{2,21} = 3.6$, $P = 0.0463$; 7 days: $F_{2,21} = 2.0$, $P = 0.1577$). * $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$ *versus* sham, † $P < 0.5$, †† $P < 0.01$, ††† $P < 0.001$ *versus* skin incision.

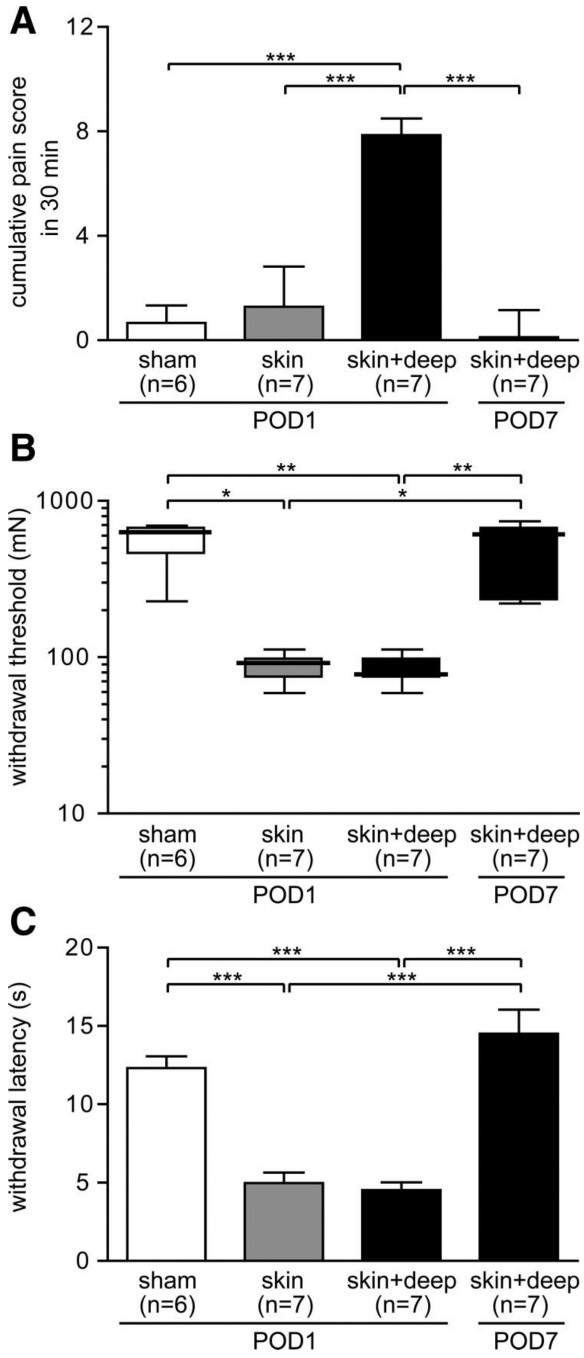

Fig. 2.

Pain behaviors of the rats that underwent electrophysiological studies. (*A*) Guarding pain behavior measured during a 30-min period. The results are presented as mean and SEM. One-way ANOVA ($F_{3,23}$ = 12.2, $P < 0.0001$) with Tukey *post hoc* test. (*B*) Withdrawal threshold to von Frey filament application. The results are expressed as median (thick horizontal line) with 1st and 3rd quartiles (box) and 10th and 90th percentiles (thin horizontal lines). Kruskal-Wallis test ($KW_{3,23}$ statistic = 21.0, $P = 0.0001$) with Dunn *post hoc* test. (*C*) Withdrawal latency to heat stimulation. The results are presented as mean and SEM. One-way ANOVA ($F_{3,23}$ = 31.5, $P < 0.0001$) with Tukey *post hoc* test. POD = postoperative day. * $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$.

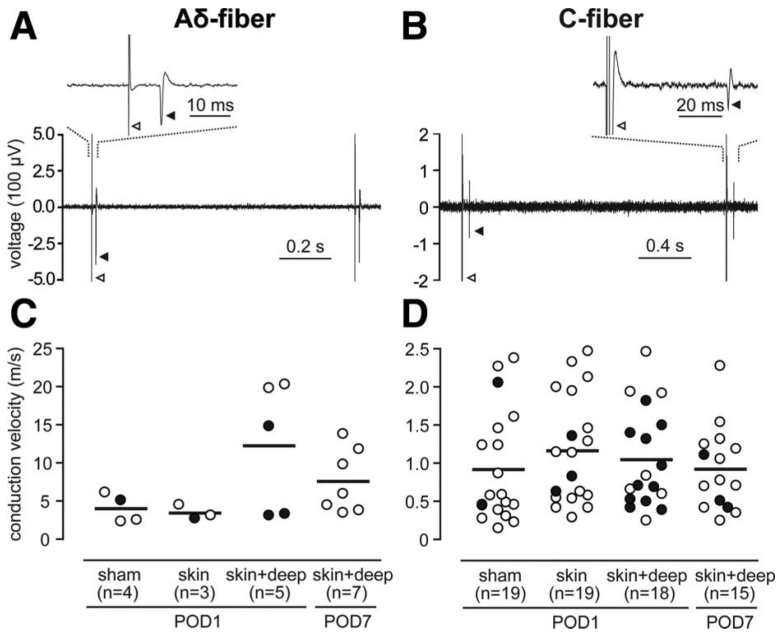

Fig. 3.

Nociceptor conduction velocities. Example of digitized oscilloscope trace of action potentials evoked by electrical stimulation at the receptive field of an Aδ-(*A*) and C-nociceptor (*B*). Open arrow head, electrical stimulation; filled arrow, evoked action potential. The distribution of conduction velocities of each Aδ-(*C*) and C-nociceptor (*D*) in each of the four groups. Open circle, nociceptors without spontaneous activity; filled circle, nociceptors with spontaneous activity. POD = postoperative day.

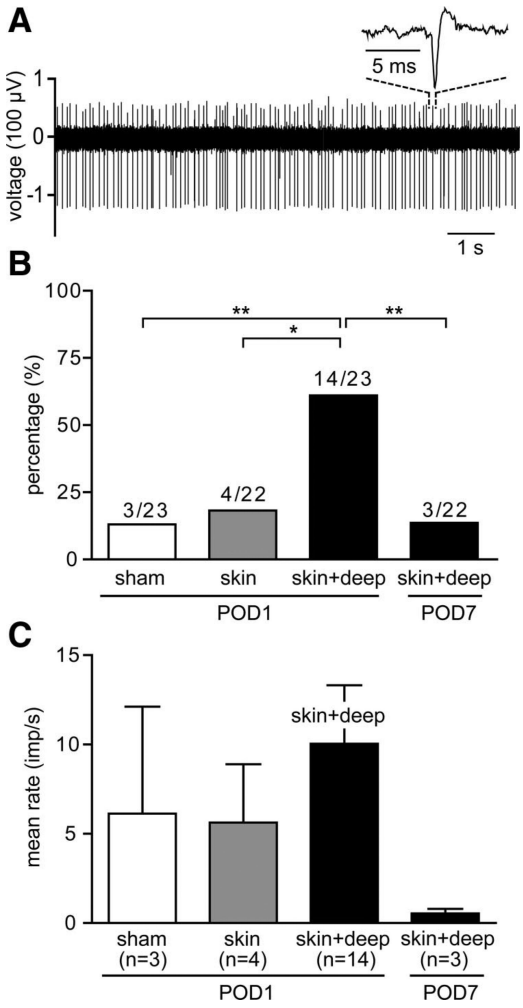

Fig. 4.

Spontaneous activity of nociceptors 1 day after sham procedure, skin, or skin plus deep tissue incision and 7 days after skin plus deep tissue incision. (*A*) Digitized oscilloscope trace of spontaneous action potentials of a C-nociceptor from a rat 1 day after skin plus deep tissue incision. Inset shows a representative single action potential. (*B*) Percentage of nociceptors with spontaneous activity in each of the four groups. Chi-square test with Bonferroni *post hoc* correction for comparisons among groups. (*C*) Comparison of average spontaneous activity rates among groups. POD = postoperative day; Imp = impulse. * $P <$ 0.5, ** $P < 0.01$.

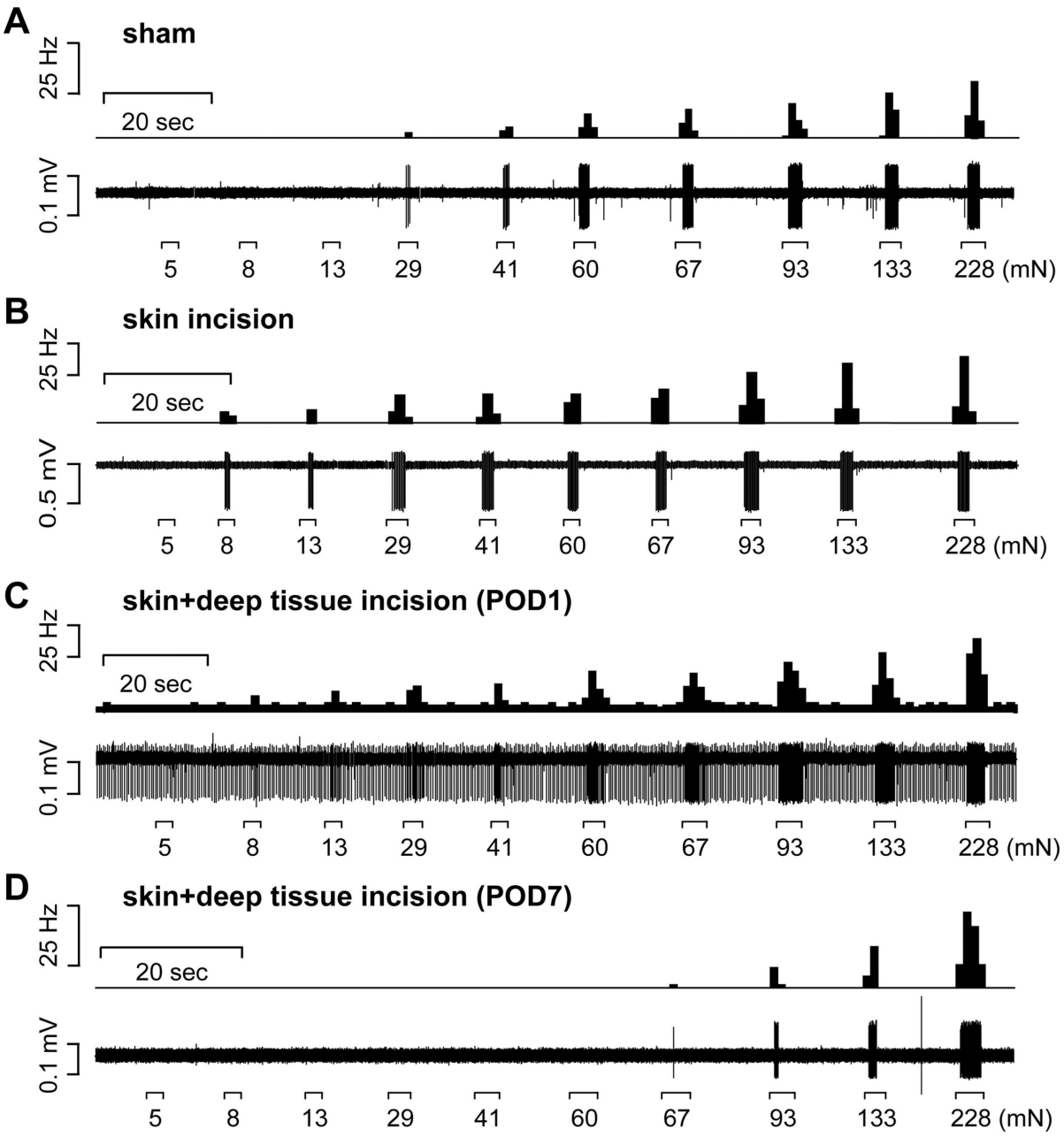

Fig. 5.

Example recordings of responses of primary nociceptors to mechanical stimulation. Digitized oscilloscope trace of action potentials evoked by von Frey filaments in the sham control (*A*), skin incision (*B*), skin plus deep tissue incision (POD1) (*C*), and skin plus deep tissue incision (POD7) (*D*) group. POD = postoperative day.

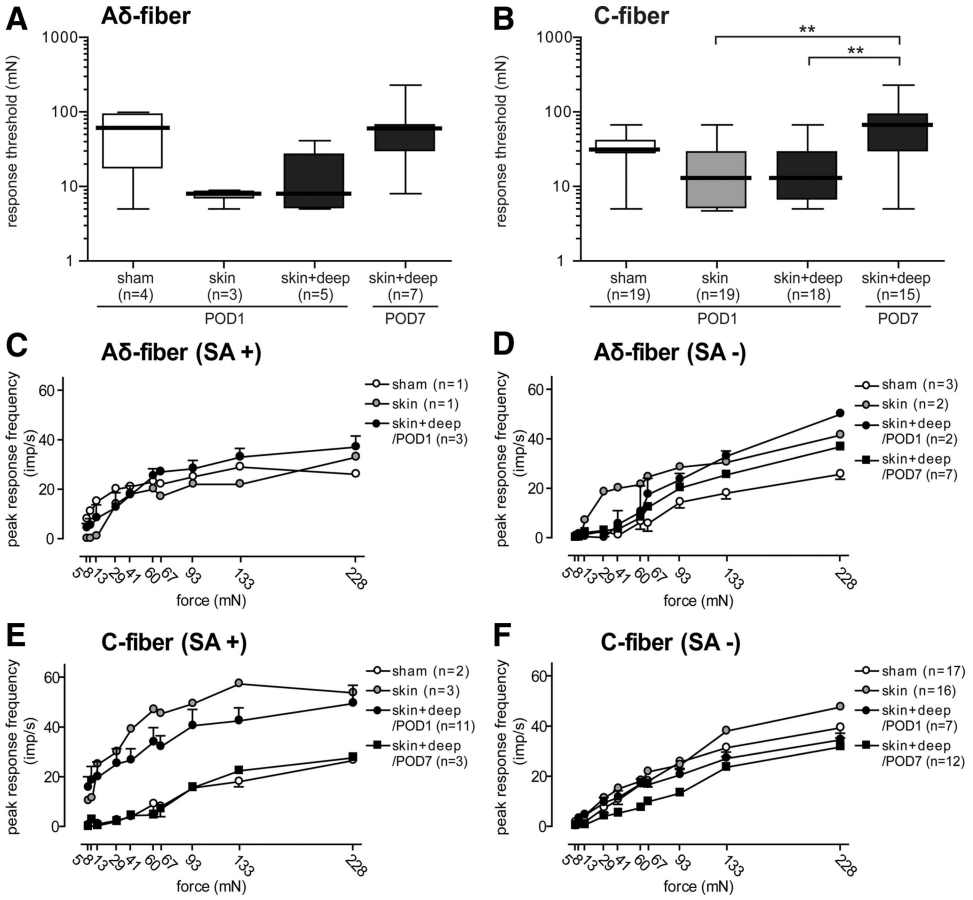

Fig. 6.

Summary of mechanical responses of nociceptors in four groups of rats. Comparison of mechanical response thresholds of Aδ-(*A*)or C-nociceptors (*B*) among the sham control, skin, skin plus deep tissue (POD1), and skin plus deep tissue (POD7) group. The results are presented as median (thick horizontal line) with 1st and 3rd quartiles (box) and 10th and 90th percentiles (thin horizontal line). Kruskal-Wallis test ($KW_{3,67}$ statistic = 13.7, P = 0.0033) with Dunn *post hoc* test for comparisons among groups. ** $P < 0.01$ *versus* the skin plus deep tissue incision group. Comparison of peak response frequency to von Frey filaments of Aδ-nociceptors with spontaneous activity (*C*) and without spontaneous activity (*D*) and C-nociceptors with spontaneous activity (*E*) and without spontaneous activity (*F*). Error bars (SEM) are shown for the sham control group and the skin plus deep tissue incision (POD1) group. Two-way ANOVA followed by separate one-way ANOVA with Tukey *post hoc* test for comparing effects of groups at each force. SA = spontaneous activity; POD = postoperative day; Imp = impulse.

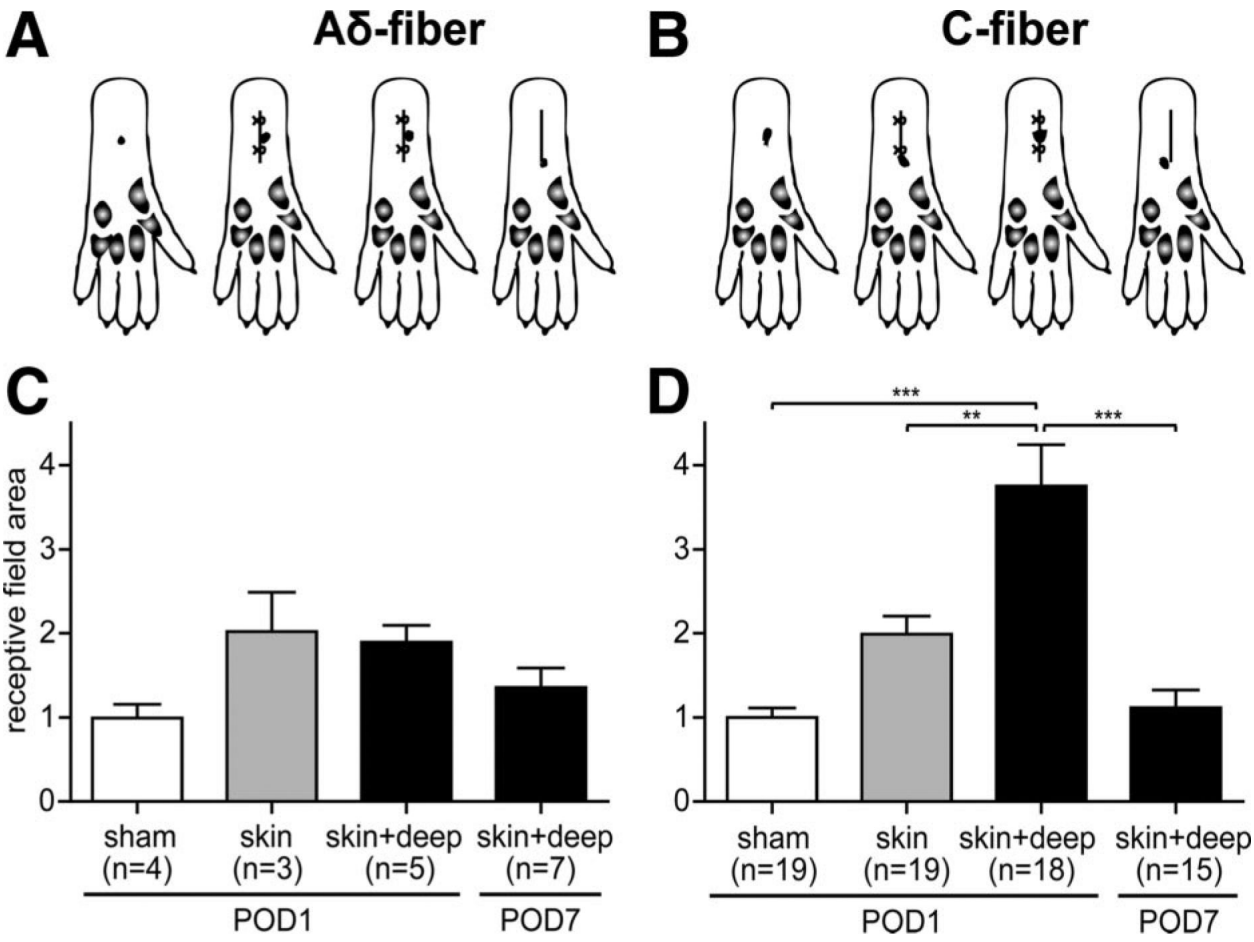

Fig. 7.

Mechanical receptive field area of nociceptors. Schematic diagrams of receptive field of Aδ- (*A*) or C-nociceptors (*B*) from the sham control, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) group. The dark area depicts the region responsive to the von Frey filament with a 240-mN bending force. The vertical line on the paw represents the location of the incision. The relative size of receptive field area of Aδ- (*C*)or C-nociceptors (*D*) from each group. The receptive field areas of the skin, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups are normalized to the receptive field area of the sham control group. The results are expressed as mean and SEM. One-way ANOVA ($F_{3,67} = 18.5$, $P < 0.0001$) with Tukey *post hoc* test for comparisons among groups. ** $P < 0.01$, *** $P < 0.001$ *versus* skin plus deep tissue incision. POD = postoperative day.

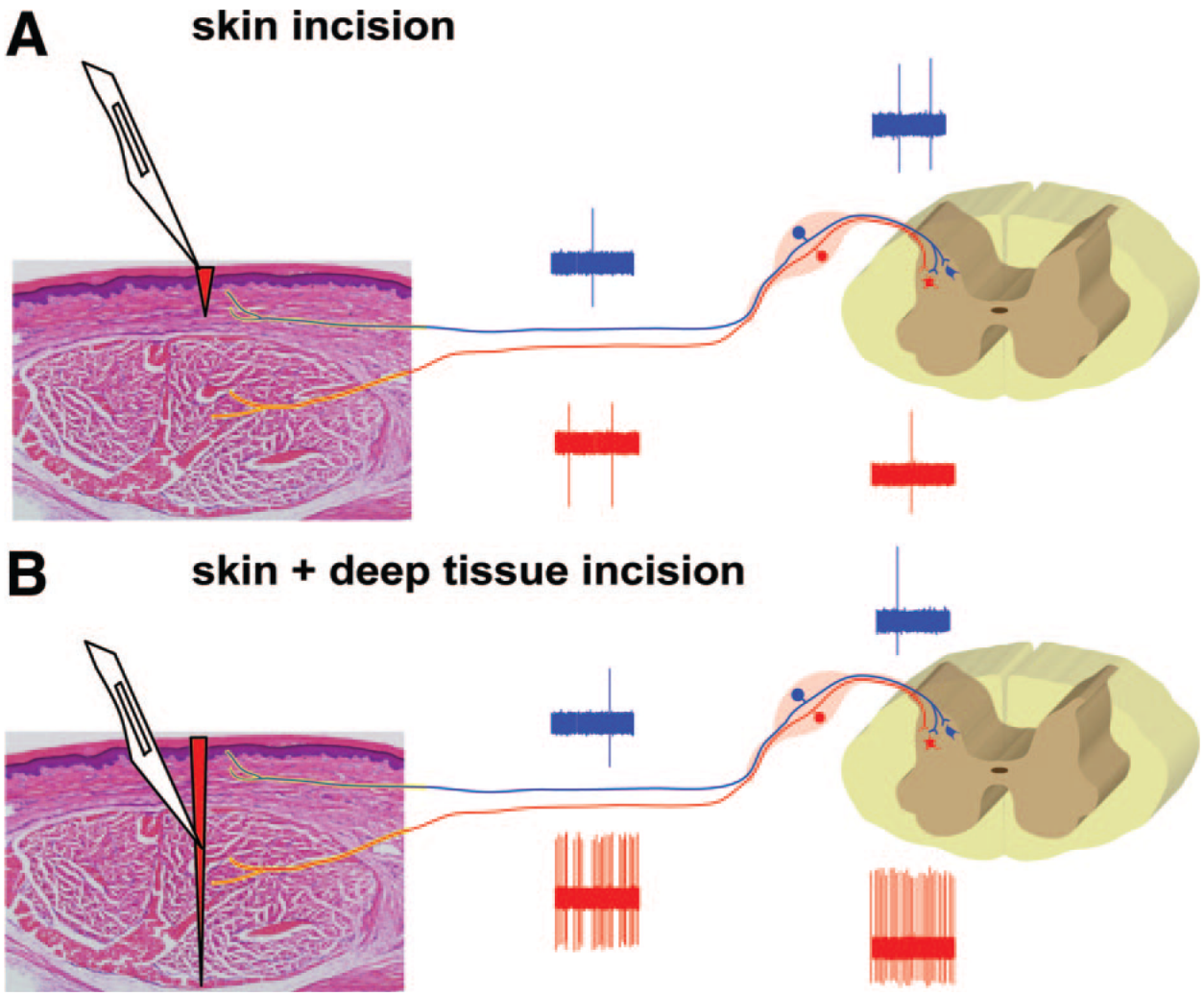

Fig. 8.

Schematic diagrams of the hypothesis that injured deep tissue rather than skin induces spontaneous activity in the nociceptive pathways. (*A*) An incision in skin only (epidermis and dermis) induces minimal spontaneous activity in nociceptors and dorsal horn neurons, which receive cutaneous input. (*B*) An incision including skin and deep tissue (fascia and muscle) produces robust spontaneous activity in muscle-innervating nociceptors and the dorsal horn neurons receiving input from the muscle.