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Comparison of skin incision versus skin plus deep tissue incision on ongoing pain and spontaneous activity in dorsal horn neurons

Jun Xu, M.D.[§] and Timothy J. Brennan, M.D., Ph.D.*

[§] Graduate Student, Departments of Pharmacology, University of Iowa Hospitals and Clinics

* Samir Gergis Professor and Vice Chair for Research, Department of Anesthesia, University of Iowa Hospitals and Clinics

Abstract

Surgery injures both skin and deep tissue causing pain at rest and evoked pain with activities. In this study, we examined the extent of injury by incision and dorsal horn neuron (DHN) spontaneous activity (SA) in rats that underwent a sham operation, skin incision or skin plus deep tissue incision. Pain behaviors were measured 1 day later followed by DHN recordings in the same rats. On POD1, guarding pain was increased in the skin plus deep tissue incision group (7.0 ± 0.7 vs. 0.1 ± 0.6 in control, $P < 0.001$), but not in the skin incision group (1.8 ± 1.0); yet, mechanical and heat hyperalgesia were similar in both incised groups. In the rats underwent skin plus deep tissue incision, more DHNs expressed SA (78.1% vs. 35.7% in control, $P < 0.01$) and SA rate also tended to be greater (13.8 ± 2.9 vs. 5.6 ± 2.0 imp/sec). Bupivacaine infiltration into the incision decreased SA in both skin incision and skin plus deep tissue incision (POD1) group to the same level as the sham operated rats. In a separate group of rats that underwent skin plus deep tissue incision, guarding pain was not present (0.1 ± 0.6) on POD7 and percentage and amount of DHN SA were the same as the sham control. These data demonstrate incised deep tissue rather than skin is critical for the development of guarding pain and increased SA of DHNs. Skin incision alone is sufficient for primary mechanical and heat hyperalgesia.

Keywords

Central sensitization; Hyperalgesia; Postoperative pain; Wide dynamic range

Introduction

Allodynia, hyperalgesia, and sensitization in nociceptive pathways have generated considerable interest in pain research. However, perhaps the most powerful form of sensitization in post injury states is spontaneous activity (SA) in nociceptive pathways, which produces unprovoked pain at rest. In postoperative patients, pain at rest or ongoing pain is a common complaint [7]. SA in the peripheral, spinal and supraspinal nociceptive pathways is likely to transmit this pain at rest after surgery.

Corresponding author: Timothy J Brennan, Department of Anesthesia, 200 Hawkins Dr. University of Iowa Hospitals and Clinics, Iowa City, IA 52242, USA. Tel: 1-319-356-7319; Fax: 1-319-356-2940; E-mail address: tim-brennan@uiowa.edu.

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In the plantar incision model for postoperative pain, unprovoked, guarding pain is evident [5]. This guarding pain is greatest immediately after incision and gradually decreases over the next 3 to 4 days. Also in this model, increased SA was identified in approximately 50% of dorsal horn neurons (DHNs) immediately and 1 to 2 hours after incision. On average, the increase in ongoing activity was from 0 to 2 imp/sec [21,23,30]. Surprisingly, when spinal DHN activity was examined 1 day after plantar incision, the SA was much greater. More than 50% of DHNs had an average activity of 20 imp/sec [26]. Moreover, during DHN recordings, administration of local anesthetic to the incision site reversed the activity of DHNs to the control or sham operated level, indicating primary afferent input maintains the increased spinal neuron activity on the day of incision or on POD1. Recordings from primary afferent nociceptors were in agreement with these DHN studies. Sustained activation of nociceptors was not demonstrated immediately after incision [11] but high SA was present in nociceptors 1 day after incision [19].

A discrepancy between behavioral results and neurophysiological data was evident. Guarding pain peaked hours after surgery and then gradually decreased on POD1 and thereafter; however, greater SA in both nociceptors and DHNs was present on POD1 compared with 1 to 2 hours after incision. There are several possibilities for this inconsistency. For instance, mechanical search strategies could bias nociceptor and DHN selection. Alternatively, perhaps there was a greater tendency to record from DHNs and nociceptors that had predominately or exclusively cutaneous input, respectively. In the present study, we hypothesized that skin incision had minimal contribution to the development of guarding pain and SA in DHNs, whereas strong guarding pain and SA could be produced by skin plus deep tissue (fascia and muscle) incision.

In this present study, rats underwent a sham operation, skin incision or skin plus deep tissue incision. Pain behaviors were measured 1 day later followed by extracellular recording of DHN activity in the same rats in. SA as well as response properties of DHNs to mechanical and heat stimuli were tested. A separate group of rats undergoing skin plus deep tissue incision was studied on POD7.

Methods

General

All experiments were reviewed and approved by The University of Iowa Animal Care and Use Committee. 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 [33]. Adult male Sprague-Dawley rats (280–320 g, Harlan, Indianapolis, IN) were used this study. Rats were housed in groups of two to three on a 12-h light/12-h dark schedule; food and water were available *ad libitum*.

Histological examination of skin incision

The thickness of rat plantar glabrous skin including the epidermal and dermal layers was estimated using histological evaluation from three rats. Animals were sacrificed with CO₂. Then under a dissecting microscope, the rat plantar hindpaw tissue which included the glabrous skin, underlying fascia and the flexor digitorum brevis muscle was harvested. The tissue was then postfixed with 4% formaldehyde in phosphate buffer for 2 days followed by incubation in 30% sucrose solution at 4°C for 2 days. Sagittal frozen sections (16 µm thickness) were cut and stained with hematoxylin and eosin (H & E). The thickness of skin was examined with an Olympus BX-51 microscope.

Based on the result from the histological examination, a blade handle was designed so that a No. 11 surgical blade (Feather Co., Osaka, Japan) could be fixed in a uniform position with

0.3 mm, 0.4 mm, or 0.5 mm of the blade tip protruding from the end of the handle. Plantar incisions were then made on 3 rats using the blade handle for each of the 3 different depths. Four hours after incision, histological samples were prepared. The 0.4 mm setting produced a plantar skin incision that traversed the skin (the epidermis and dermis) without cutting the deep tissue (fascia and muscle layers).

Surgical incisions

A total of 50 rats, divided into 4 groups, were used for behavioral and subsequent electrophysiological studies. Anesthesia was induced by placing the animal in a sealed plastic box with 5% isoflurane mixed with air. During the following surgical procedures, anesthesia was maintained at 1.5–2% isoflurane delivered via a nose cone to the animal. The left hindpaw was prepared in a sterile manner with 10% povidone-iodine. Twelve rats were subjected to skin incision only. For skin incision, beginning 0.5 cm from the proximal edge of the heel, a 1-cm longitudinal incision was made with the designed blade handle as mentioned above. During the incision, the blade handle was applied against the plantar surface indenting the skin to make the skin incision. The incised skin was then opposed with three subcutaneous mattress sutures with 6-0 nylon on a P-1 needle (Ethicon, Somerville, NJ) while visualized under a binocular dissecting microscope. The wound was covered with antibiotic ointment.

Twenty seven rats underwent skin plus deep tissue incision. These rats were studied on POD1 (skin+deep, n=14) or on POD7 (skin+deep, n=13). The skin plus deep tissue incision was made similarly to that described previously (Brennan et al. 1996). At the same plantar site as in the skin incision, a 1-cm longitudinal incision was made through the skin, underlying fascia and the plantar flexor digitorum brevis muscle with a No. 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 as described previously [5] 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. Eleven rats underwent a sham procedure, which included isoflurane anesthesia, sterile preparation of the hindpaw, and topical antibiotics, but no incision was made. For the POD7 rats, sutures were removed on POD2. The person performing the behavioral and electrophysiological studies was blinded to type of incision on POD1 but could not be blinded to sham or POD7 incision.

Behavioral studies

One day after sham, skin, or skin plus deep tissue incision, the rats underwent three types of pain behavior tests: guarding pain behavior, withdrawal threshold to punctate mechanical stimuli and withdrawal latency to radiant heat, described in detail [5,29]. Another skin plus deep tissue incision group was evaluated on POD7. To lessen the time to begin the electrophysiology studies, the pain behavioral tests were abbreviated. The guarding pain was decreased from 60 min to 30 min and only one mechanical and heat tests (rather than three) were performed.

For guarding behavior [5], unrestrained rats were placed individually on a small plastic mesh floor (grid 8×8 mm) covered with a plastic chamber (21×27×15 cm). Using an angled magnifying mirror, the position of the rat hindpaw was closely observed during a 1-min period repeated for every 5 min for 30 min. Based on the hindpaw position during the majority of 1-min observation period, a score of 0, 1 or 2 was given. Zero was given for full weight bearing position of the incised area of the hindpaw which was blanched or distorted by the mesh; 1 was given if the incised area of the hindpaw just touched the mesh without blanching or distortion; 2 was given if the incised area of the hindpaw was completely off the mesh. The 6 scores during the 30 min session were added together (0–12) for each paw. Then the guarding

pain score was determined by subtracting the sum score of incised hindpaw from that of non-incised hindpaw. The maximum score was 12 rather than 24.

For withdrawal threshold to punctate mechanical stimuli [5], rats were placed individually on a plastic mesh floor (grid 12×12 mm) covered with a plastic chamber (21×27×15 cm). Calibrated von Frey filaments were applied from underneath the mesh to an area adjacent to the incision or a corresponding area in sham rats. Each filament was applied once starting with a bending force of 13 mN and continued until a withdrawal response was evoked or a bending force of 228 mN was reached. If a rat did not respond to the 228 mN filaments, the next filament (609 mN) was recorded as threshold. The force eliciting a withdrawal response was taken as the mechanical withdrawal threshold. Only one withdrawal response was measured.

For withdrawal latency to radiant heat stimulation [29], rats were placed individually on glass floor covered with a clear plastic chamber (21×27×15 cm). A focused radiant heat source was applied from underneath the glass floor and targeted to the incision site or a corresponding area in sham rats. The latency (in sec) to evoke a withdrawal response was recorded. Only one withdrawal latency was measured.

Neurophysiological studies

General—After the behavioral tests, rats underwent *in vivo* extracellular DHN recording. The surgical procedures were performed according to the previous methods [21,30]. Briefly, anesthesia was initially induced with 5% isoflurane in air through a sealed box and then maintained with 2% isoflurane in air via a nose cone during the following surgery procedures. The common carotid artery was cannulated to monitor the blood pressure. Then a tracheotomy was performed and the rat trachea was connected to a ventilator (Harvard Apparatus, Inc, South Natick, MA) via a tracheal cannula. The animal was artificially ventilated with oxygen and anesthesia was maintained with 2% isoflurane. The rat was then positioned in a stereotactic frame. The head and vertebral column of the rat were stabilized with ear bars and vertebral clamps, respectively. Limited laminectomies were performed to expose the dorsal spinal cord at the lumbar enlargement between 13th thoracic and 3rd lumbar vertebra. The underlying dura was removed and the spinal cord was covered with mineral oil. The left dorsal hindpaw was imbedded into a small block of clay and the plantar aspect was exposed for testing.

After these surgical procedures, anesthesia was decreased to 1.5% isoflurane during the subsequent recording period. For DHN recording, small holes were made with fine forceps on the pia mater of the left side lumbar enlargement between the L3 and L6 segments. A tungsten parylene-coated electrode (1–1.5 mΩ impedance, Microprobe Inc., Clarksburgh, MA) was then driven into the dorsal spinal cord through the pia mater hole. Neuron activities were amplified (Grass Instruments, Quincy, MA), displayed on an oscilloscope and discriminated on the basis of amplitude and waveform (BAK Electronics, Germantown, MD). All data was recorded and stored into a PC computer with a data acquisition system, 1401 Plus Laboratory Interface and Spike2 software (Cambridge Electronic Design, Cambridge, UK).

During the DHN recording, the mean arterial blood pressure was at least 90 mmHg. The body temperature was maintained at approximately range 35–37°C with a servo-controlled electric heating lamp and an underbody heating pad. Arterial blood gases were measured periodically and the ventilation rate was adjusted correspondingly to maintain arterial carbon dioxide between 35 and 45 mmHg.

Recording protocol—The recording electrode was lowered to the surface of the spinal cord onto the pia mater hole and the depth of the electrode was set as 0 μm. Then the electrode was advanced into the spinal cord slowly at 10 μm per step via a micropositioner (David kope instruments, Tujunga, CA) until a neuron with ongoing SA was encountered or a depth of 1000

μm was reached. If a neuron with SA was detected, the mechanical receptive field (RF) of the neuron was determined by applying mechanical stimulation (tapping and a 228 mN von Frey filament) to the plantar hindpaw. The neuron was accepted for further study only if its mechanical RF included at least part of the incision site. If no neurons met the criteria, and the 1000 μm depth was reached, the electrode was then driven backwards to the dorsal surface of the spinal cord at a rate of 10 $\mu\text{m}/\text{step}$ via the micropositioner while touching and tapping were applied to the distal plantar hindpaw. Repeated stimulation was used to search for DHNs that received input from the plantar hindpaw that did not necessarily have SA. Again these neurons without SA were accepted for further study if the RF included at least part of the incision site. The depth of the neuron recorded was based on the electrode position optimized for the action potential amplitude.

After a neuron with a RF that included the incision site was identified using either search stimulus (SA or mechano-evoked activity from the hindpaw), a baseline measurement for ongoing activity was recorded for 10 min. SA of the neuron during the last 5-min period was averaged. A neuron with a mean activity rate greater than 0.1 Hz (a minimum of 30 impulses during the 5-min period) was considered spontaneously active.

Following the measurement of SA, brush (camel hair brush No. 4) was applied to the entire hindpaw and the response of the neuron was measured. Next, the mechanical RF area and the most mechanosensitive spot of the neuron were determined using a von Frey filament with a bending force of 228 mN and drawn on a schematic of the plantar hindpaw. The relative size of RF area was measured with the NIH Image J software from a scanned image of the drawing.

To characterize the punctate mechanical response properties, following the RF area measurement, calibrated von Frey filaments with bending forces of 5, 8, 13, 29, 41, 60, 67, 93, 133, and 228 mN were applied in an ascending order to the most mechanosensitive spot in the RF. Forces greater than 228 mN were not applied to avoid potential injury to the RF. Each of the von Frey filaments was applied to the RF on the hindpaw, bent, held in this position for 3 to 5 seconds, and removed. The interstimulus interval was 5–10 sec. By using 1 sec bin width, the peak response frequency of a neuron was determined as the greatest rate during the filament application. A stimulus-response function was then generated by plotting the peak response frequency (imp/sec) during a filament application versus the force of each filament. The mechanical response threshold of each neuron was defined as the lowest force that caused either activation of the neuron if no SA was present or an increase in neuron activity by at least two standard deviations above mean SA before the mechanical stimulation [23]. The next strength filament must also have excited the neuron. Then a non tissue damaging pinch was applied with a blunt curved forceps.

Both wide dynamic range (WDR) and high threshold (HT) neurons were included. The classification was based on response of the neuron to brush and to pinch. HT neurons were identified when neurons only responded to pinch but not to brush; WDR neurons responded to brush and had an even greater response to pinch. If any low threshold cells were encountered (responding maximally to brush but had the same or smaller response to pinch), these neurons were not studied. Because all neurons were recorded after incision (or sham), it is possible that HT neurons were converted to WDR neurons. Neurons were referred as WDR and HT because in four previous studies we could not convert an HT to a WDR neuron with incision [21,23, 28,30] and the proportion of HT and WDT neurons is similar to previous studies in unincised rats.

To examine heat response properties of DHNs, following the mechanical stimuli, a standard heat ramp was applied to the RF using a feedback-controlled Peltier device (Yale. Instrumentation, New Haven, CT). The surface of the Peltier device (contact area 1 cm^2) was

connected to a manipulator and positioned to contact the mechanical RF on the plantar hindpaw. A baseline temperature (32 °C) was applied to the hindpaw for 3 min. This was followed by a standard heat ramp from 32 to 48 °C in 14 sec. The temperature was held at 48 °C for 2 sec and then decreased to 32 °C over 14 sec. The heat responsiveness was defined as: presence of evoked neuron activities by heat stimulation if no SA was present before heat stimulation, or an increase in neuron activity during the heat stimulation by at least two standard deviations above the mean SA before heat stimulation. The peak response frequency (1 sec bin width) of a neuron was measured as the greatest rate during the heat application. The total action potentials during the 14 sec heat ramp and through the 1 sec after the peak temperature were counted. The SA was included in the total discharge measurement as for mechanical responses. The heat response threshold of each neuron was defined as the lowest temperature that caused either activation of the neuron if no SA was present or an increase in neuron activity by at least two standard deviations above mean SA before the heat stimulation.

Bupivacaine injection

The contribution of peripheral input to the spontaneous activities in the DHNs was further determined by local injection of bupivacaine (Hospira, Lake Forest, IL) into the incision site in the incised rat or the corresponding site in the sham control rat. Neurons with SA were selected in the bupivacaine test and they were the last neurons recorded from the preparation. First, SA was recorded for 5 min. Then, the incision site was anesthetized by injection of 0.5% bupivacaine (0.3 ml) subcutaneously and into the deep muscle tissue. Five min later, the SA was measured for the next 5 min. Finally, the von Frey filament with a bending force of 228 mN and pinch were applied to the RF again to assess blockade by the local anesthetic. For some neurons, additional bupivacaine was injected to assure the effect of the incision on activity was eliminated. This additional drug did not have any further affect on the activity. A decrease in activity by bupivacaine was defined as a decrease in neuron activity by at least two standard deviations from the activity 5 min before the injection.

Statistical analysis

For continuous data, the Kolmogorov-Smirnov test of normality was used to determine whether the data values have normal (Gaussian) distributions. One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to analyze the guarding pain behavior, withdrawal latency to heat, the rate of SA, RF area of HT and WDR neurons, total discharge and peak discharge rates during heat stimulation, and heat response threshold. Kruskal-Wallis test with Dunn's post hoc test was used for withdrawal threshold to mechanical stimulation. Two-way ANOVA followed by repeated one-way ANOVA was performed to analyze the mechanical stimulus-response functions. χ^2 -test with Bonferroni correction for multiple tests was used to compare the percentage of WDR vs HT neurons, neurons with and without SA and neurons responding to heat among groups. Student's t-test was made to compare the depth of neurons with and without SA in the spinal cord. Two-way ANOVA followed by repeated one-way ANOVA and paired t-test was used to evaluate the effect of bupivacaine on spontaneous activities. Values of $P < 0.05$ were considered significant. All data are presented as mean $\pm$ standard error of the mean (SEM), unless otherwise stated. All tests were performed with GraphPad Prism software (GraphPad, San Diego, CA).

Results

Effect of incisions on hindpaw tissue

The skin incision cut the epidermis and dermis layer without injuring the underlying fascia and muscle tissue. One day later, the incised epidermis and dermis were well-apposed (Fig. 1B–C). Swelling and inflammation were present in the superficial fascia. Incision in the skin plus deep tissue divided the plantar hindpaw from the superficial epidermis to the deep flexor

digitorum brevis muscle. One day later, the wound was apposed (Fig. 1D–E). The deep tissue incision induced surrounding inflammation and swelling through the muscle. Seven days after incision of the skin plus deep tissue, the wound showed fibrosis, which was evident in the dermis, fascia and muscle tissue (Fig. 1F).

Behavioral studies

The cumulative guarding pain score was 0.8 ± 0.6 , 1.83 ± 1.0 , 7.0 ± 0.7 and 0.1 ± 0.6 in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) groups, respectively (Fig. 2A). The guarding pain score of the skin plus deep tissue incision (POD1) group was greater than that of the sham group ($P < 0.001$). On POD7, the skin plus deep tissue incision group was not different than the sham group. The guarding pain score of the skin plus deep tissue incision (POD1) group was also greater than that of the skin incision ($P < 0.001$) and skin plus deep tissue incision (POD7, $P < 0.001$) group.

For mechanical responses, the median withdrawal threshold to mechanical stimulation was 673mN, 73 mN, 73mN and 673 mN in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) groups, respectively (Fig. 2B). Both the skin incision group and skin plus deep tissue incision (POD1) group had lower mechanical thresholds than the sham group ($P < 0.001$); there was no difference between the sham and skin plus deep tissue incision (POD7) groups.

For heat responses, the withdrawal latency to heat was 12.8 ± 0.6 , 4.5 ± 0.2 , 3.5 ± 0.2 and 13.9 ± 0.9 sec in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7), respectively (Fig. 2C). Both the skin incision group and the skin plus deep tissue incision (POD1) group had lower heat withdrawal latencies than the sham group ($P < 0.001$); there was no difference between the sham and the skin plus deep tissue incision (POD7) groups.

Electrophysiological studies

Extracellular single-unit activities were recorded from a total of 124 DHNs in 50 rats; these neurons were classified into HT and WDR neurons: 28 neurons (8 HT and 20 WDR) in the sham group, 30 (3 HT and 27WDR) in the skin incision group, 32 (6 HT and 26 WDR) in the skin plus deep tissue incision (POD1) group, and 34 (10 HT and 24 WDR) in the skin plus deep tissue incision (POD7) group. The numbers of HT and WDR neurons were not different among groups. All of the neurons had a mechanical RF in the plantar hindpaw, part of which included the incision or the corresponding site in the unincised hindpaw. No more than 3 neurons were recorded from each rat.

Spontaneous Activity

The percentage of neurons with SA was 35.7%, 53.3%, 78.1% and 29.4% in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) groups, respectively (Fig. 3B). The skin plus deep tissue incision (POD1) group had significantly more neurons with SA than the sham group ($P < 0.01$); the sham group had a similar percentage of SA neurons as the skin incision and skin plus deep tissue incision (POD7) groups. The skin plus deep tissue incision (POD1) group also had more neurons with SA than skin plus deep tissue incision (POD7, $P < 0.001$).

The mean rate of SA was 5.6 ± 2.0 imp/sec, 8.8 ± 1.5 imp/sec, 13.8 ± 2.9 imp/sec, 5.8 ± 2.1 imp/sec in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) groups, respectively (Fig. 3C). The rate of the skin plus deep tissue incision (POD1) group was the greatest, but no significant differences were found among groups.

Depth of DHNs

The depths of neurons with and without SA were compared within each group. The number of HT neurons was small, limiting the interpretation of the statistical analyses. For HT neurons, no significant differences in depth of SA neurons vs. neurons without SA was present within the 4 groups (Fig. 4A).

For WDR neurons, the mean depths of neurons with and without SA were $650.0 \pm 92.9 \mu\text{m}$ vs. $613.8 \pm 78.7 \mu\text{m}$, $751.5 \pm 65.2 \mu\text{m}$ vs. $591.4 \pm 61.6 \mu\text{m}$, $748.1 \pm 43.8 \mu\text{m}$ vs. $404.0 \pm 33.0 \mu\text{m}$ ($P = 0.001$), and $741.3 \pm 43.3 \mu\text{m}$ vs. $568.1 \pm 41.4 \mu\text{m}$ in the ($P = 0.016$) in the sham, skin incision, skin plus deep tissue incision (POD1), and skin plus deep tissue incision (POD7) groups, respectively (Fig. 4B). Therefore, 1 day after skin+deep tissue incision, more neurons had SA, the activity tended to be greater in magnitude and these neurons were located deeper in the spinal cord than those without SA. Seven days later, a tendency for those with SA to be deeper remained although the proportion of neurons and the magnitude of SA were not different than sham.

Effect of bupivacaine on SA

Bupivacaine injections were made while recording SA from neurons of rats that underwent sham ($n=5$), skin incision ($n=8$), skin plus deep tissue incision ($n=9$, POD1) and skin plus deep tissue incision ($n=7$, POD7), respectively. Before bupivacaine injection, the rate of SA was 7.0 ± 1.5 imp/sec, 13.6 ± 3.4 imp/sec, 21.9 ± 3.8 imp/sec and 7.4 ± 2.4 imp/sec in the 4 groups, respectively (Fig. 5E). The rate of SA of the skin plus deep tissue incision (POD1) group was greater than that of the sham group ($P < 0.05$); the rates of skin incision and skin plus deep tissue incision (POD7) group were not different than that of the sham group. We selected DHNs with SA for bupivacaine injection late the experiment, which might have resulted in a higher average SA compared to the rate of all neurons (Fig. 3C).

Bupivacaine injection decreased SA in 0 out of 5, 7 out of 8, 7 out of 9, and 2 out of 7 neurons in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) group, respectively. A greater percentage of neurons were inhibited by bupivacaine injection after skin incision ($P < 0.05$) and after skin plus deep tissue incision (POD1, $P < 0.05$) than after sham. After bupivacaine injection, the rate of SA was 6.7 ± 1.9 imp/sec, 5.3 ± 1.4 imp/sec, 10.9 ± 3.5 imp/sec and 6.0 ± 2.6 imp/sec in the 4 groups, respectively. There was no difference in the rates of SA among groups after bupivacaine injection. Within each group, bupivacaine injection significantly reduced the average SA rate of the skin incision ($P < 0.05$) and skin plus deep tissue incision (POD1) groups ($P < 0.05$), but had no effect on the average SA rate of the sham and skin plus deep tissue incision (POD7) groups.

Response to heat

Heat was studied in most neurons: 27, 26, 25 and 33 neurons in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) groups, respectively. Typical examples of heat responsive neurons in each of the 4 groups were shown in Fig. 6A–D.

Overall, 19%, 31%, 32% and 48% of neurons innervating the incision responded to heat in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) group, respectively (Fig. 6E). No significant difference in percentage of responsive neurons was present among groups. The total discharges and peak response frequencies during the heat simulation were also not significantly different among groups (Fig. 6F–G). The heat response threshold was $43.2 \pm 0.3^\circ\text{C}$, $40.6 \pm 0.7^\circ\text{C}$, $40.0 \pm 0.9^\circ\text{C}$ and $45.5 \pm 0.5^\circ\text{C}$ in the sham, skin incision, skin plus deep tissue incision (POD1) and the skin plus deep tissue incision

(POD7) groups, respectively (Fig. 6H). The heat threshold of the skin plus deep tissue incision group (POD1) was less than that of the sham ($P < 0.05$) and skin plus deep tissue incision groups (POD7, $P < 0.001$). There was no difference in dorsal horn depth of heat responsive vs. nonresponsive DHNs among the incised groups (data not shown). In all groups, there was no significant relation between SA and heat threshold, total action potentials and peak rate during heat stimulation for neurons with and without SA, perhaps because the number of heat responsive neurons was small.

Responses to mechanical stimuli

The most mechanosensitive spot on the RF was most often distal to the incision near the tori of the hindpaw. DHNs with and without SA exhibited increased responses to greater filament forces ($P < 0.001$). The force-activity relation was less remarkable in neurons with SA than neurons without SA (Fig. 7B, D). This different force-activity relations between neurons without SA and neurons with SA was also noted previously in sham and incised rats (Xu et al. 2008).

Differences in force-activity relations among the 4 groups were found in neurons without SA ($P < 0.01$) but not in neurons with SA. For those without SA, both skin plus deep tissue incision groups (POD1 and POD7) were more responsive to higher forces than both the sham and skin incision groups (Fig. 7B). No difference was found between the sham and skin incision groups.

Based on the limited number of neurons, HT neurons also showed increased responses to greater forces but only at the higher strengths ($P < 0.001$). There was no difference in force-activity relations among the 4 groups (data not shown).

Examples of typical areas of the mechanical RF of WDR neurons are shown in Fig. 7E. The normalized RF size was $100 \pm 9\%$, $139 \pm 16\%$, $190 \pm 14\%$ and $44 \pm 8\%$ in the sham, skin incision, skin plus deep tissue incision (POD1) and skin plus deep tissue incision (POD7) groups, respectively (Fig. 7F). The RF size of the skin plus deep tissue incision (POD1) group was greater than that of the sham group ($P < 0.001$), the skin incision group ($P < 0.05$) and the skin plus deep tissue incision (POD7) group ($P < 0.001$).

Discussion

The present study, for the first time, examined the contributions of skin versus skin plus deep tissue incision to a variety of pain-related behaviors and characterized DHN activity from the same rats. Skin plus deep tissue incision caused guarding pain 1 day later whereas skin incision did not. However, both types of incisions resulted in similar mechanical and heat hyperalgesia. After skin plus deep tissue incision, more DHNs expressed SA and for those neurons with SA, greater firing rates were also suggested. Primary afferent input maintained the ongoing spinal neuron activity on POD1. Seven days later, guarding pain produced by skin plus deep tissue incision had resolved, and SA of DHNs was the same as that in sham operated rats.

Incision and guarding pain behavior

We have previously characterized guarding pain behavior after plantar incision and suggest it is a correlate to pain at rest in postoperative patients. Previous studies demonstrated low doses of parenteral morphine (0.03–0.1 mg/kg), local anesthetic infiltration, and NGF sequestration reduced the guarding pain behavior [3,21,25]. In these studies, the effects of these drugs were modality specific. The lowest dose of morphine affected guarding pain only [25]; NGF sequestration decreased guarding and heat but not mechanical responses [3,31].

Skin incision and SA in DHNs

An important finding of the present study is that incision of skin alone produced minimal guarding pain on POD1 and a modest amount of SA in DHNs. The SA after skin incision was not significantly greater than that in sham. These data indicate a minor role of incised skin in guarding pain and DHN SA compared to skin plus deep tissue incision.

In agreement, incision in hairy skin of the rat hindquarter induced only weak, transient SA in DHNs [12]. In humans, a 4-mm long incision through the skin in the volar forearm caused pain at rest for less than 30 min [13,14].

Skin plus deep tissue incision and SA in DHNs

The current study revealed a strong relationship between SA of DHNs and guarding pain behavior. In agreement, previous studies showed that intrathecal non-N-methyl-D-aspartate (NMDA) receptor antagonists decreased guarding pain and spontaneous DHN activity induced by plantar incision [30,32]. Conversely, spinal NMDA receptor blockade did not inhibit guarding pain or decrease SA of DHNs after incision [27,30].

Guarding pain behavior is greatest immediately after plantar incision, slightly less on POD1 and resolves by POD3 or 4 [5]. However, in previous studies, the immediate effect of plantar incision within the RF of DHNs resulted in small increases in SA 1 to 2 hours later [21,23,30]. The average increase 1 hour after incision was usually only 2 imp/s, much less compared to the robust activation seen on POD1 (Xu et al., 2008 and the present study), when guarding pain is less. We hypothesize this discrepancy is likely related to neuron selection. When the immediate effect of plantar incision on DHNs was examined in previous studies, the search stimulus was touching and tapping to the hindpaw. Thus, the response to mechanical stimulation was the primary search criteria. Neurons receiving predominantly skin input and relatively less input from deep tissues might have been selected.

Consistent with previous data [26], the present study showed that local anesthesia reversed the high SA in skin plus deep tissue incision to the same post-injection level as that in the sham operated rat. In support, nociceptors with high spontaneous firing rates were evident 1 day after incision in skin plus deep tissue [19] indicating that high nociceptor activity is present after skin, fascia and muscle incision. These data suggest that deep tissue nociceptors generate much greater SA after incision than incised cutaneous nociceptors. In agreement, using electrical stimulation, muscle nociceptors produced greater DHN activation and central sensitization than cutaneous nociceptors [16,24].

After skin plus deep tissue incision, neurons with SA were deeper in the spinal dorsal horn than neurons without SA. SA from neurons in deeper lamina may occur because deep tissue afferents terminate in deeper lamina of the spinal cord [2,4,6]. The SA could also be intrinsic to deep DHNs. As suggested by Derjean et al. (2003), deep DHNs have three firing modes: normal tonic discharge, plateau potentials, and spontaneous rhythmic discharge. Most (90%) deep DHNs have intrinsic properties through which peripheral afferent activity can switch these DHN firing patterns. Thus, sustained DHN SA could develop if the rhythmic discharge mode is induced by nociceptor activation [8].

Heat and mechanical responses

Heat withdrawal latency was similarly reduced by skin incision and skin plus deep tissue incision. This demonstrates skin incision alone is sufficient for development of heat hyperalgesia 1 day after incision. Consistent with the behavioral data, both skin incision and incision of skin plus deep tissue resulted in lower heat thresholds of DHNs.

Mechanical withdrawal threshold was similarly reduced by skin incision and skin plus deep tissue incision. The finding that skin incision alone is sufficient to induce mechanical hyperalgesia agrees with other studies. Primary mechanical hyperalgesia was induced for several days by skin incision in humans [13,14] and by incision in rat hairy skin [10,12].

When mechanical responses of WDR neurons were examined, neurons without SA showed enhanced mechanical responses, while neurons with SA had only weak responses to mechanical stimuli. Consistent with our previous study [26], these data suggest separate functions of these two groups of DHNs after incision, one group with SA and weak responses to mechanical stimuli and one group without SA and responsive to mechanical stimuli. Alternatively, it may also be difficult to further excite neurons with mechanical stimuli if SA is high.

Behavioral studies demonstrated similar withdrawal thresholds after skin and after skin plus deep tissue incision. However, compared to the sham group, only skin plus deep tissue incision but not skin incision sensitized WDR neurons to mechanical stimulation (Fig. 7). This discrepancy may be related to the test site. A site immediately adjacent to the incision was chosen for withdrawal threshold in behavioral studies, whereas the most mechanically sensitive site of the hindpaw was tested for the stimulus-response functions of DHNs. In most cases, the test sites were located distal and outside the incision (Fig. 7). Thus, the increased mechanical responses of DHNs after skin plus deep tissue incision likely reflects secondary hyperalgesia or referred pain in behavioral studies [20,29]. Secondary, distal hyperalgesia is robust after skin, fascia and muscle incision of the hindpaw.

Clinically, postoperative patients note pain evoked by touching both the wound site and the surrounding non-injured area. The present behavioral and electrophysiological results indicate that incision in the skin alone is sufficient to induce primary hyperalgesia, however, exaggerated responses of DHNs to uninjured areas of the RF were not evident after skin incision.

The mechanical RF size of WDR neurons after skin plus deep tissue incision was also greater than that after skin incision. The RF that incorporates unincised areas is a result of central sensitization [22]. Together, these data imply stronger central sensitization, measured by mechanical responses distant to the incision and the size of the RF, occurs after skin plus deep tissue incision compared to skin incision alone.

The presence of increased mechanical responses on POD7 suggests prolonged DHN sensitization following incision of skin plus deep tissue. It agrees with a previous study showing decreased mechanical withdrawal threshold in an area 1-cm distal to the incision was present 7 days after skin plus deep tissue incision [29].

Clinical implications

In our model, deep tissue rather than skin is critical for guarding pain and DHN activation after incision. In support of this concept, two surgical approaches for a unilateral total hip arthroplasty, a minimally invasive approach and a conventional approach, were recently compared [9]. Both approaches used the same length of skin incision, 20 cm. The minimally invasive approach preserved the underlying muscles whereas muscles were incised and divided in the conventional approach. The minimally invasive approach resulted in significantly less postoperative pain than the conventional approach. In a complimentary study [17], there was no difference in postoperative pain between groups when the same degree of deep tissue injury occurred through different lengths of skin incision.

Our incision model does not cause persistent pain which, however, is present in 10–50% patients 3–6 month after surgery [15]. Major nerve injury during surgery is thought to result in such persistent pain [1,15,18] and to our knowledge this does not occur in our model.

Conclusion

Our data demonstrate that deep tissue rather than skin incision is critical for the development of spontaneous guarding pain. The present data further demonstrate a strong relation between spontaneous DHN activity and guarding pain. Peripheral nociceptor input maintains robust spinal DHN activity after incision. Incision of skin plus deep tissue results in greater DHN sensitization as measured by mechanical RF size and mechanical stimulus-responses generated remote to the incision. Finally, skin alone is sufficient to induce primary mechanical and heat hyperalgesia after incision. The present study also suggests that after surgery, the injured deep tissue is an important target for both clinical pain management and new drug development.

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Figure 1.

H & E stained sagittal sections of the normal and incised rat hindpaw including the grabrous skin to the plantar flexor digitorum brevis muscle. (A) Normal rat hindpaw. (B) Four hours after skin incision. (C) One day after skin incision. Histology of rat hindpaw with skin plus deep tissue incision, 4 h (D), 1 d (E), and 7 d after the incision (F). Arrows, the site of incision in skin or skin plus deep tissue incision. Arrow heads, the superficial wound. All scale bars represent 500 μ m. Seven days after the incision, granulation tissue (empty arrow heads) is present in the dermis, fascia and muscle around the incision site.

Figure 2.

Pain behaviors of rats that underwent DHN recording 1 day after sham, skin, or skin plus deep tissue incision and 7 days after skin plus deep tissue incision. (A) Guarding pain behavior measured during a 30-min period. The results are presented as mean and SEM. *** $P < 0.001$, one-way ANOVA, Tukey's 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). *** $P < 0.001$, Kruskal-Wallis test, Dunns post hoc test. (C) Withdrawal latency to heat stimulation. The results are presented as mean and SEM. *** $P < 0.001$, one-way ANOVA, Tukey's post hoc test.

Figure 3.

SA of DHNs 1 day after sham, 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 WDR neuron from a rat that underwent skin plus deep tissue incision (POD1). Inset shows a representative single action potential. (B) Percentage of DHNs with SA. ** $P < 0.01$, ***, $P < 0.001$, χ^2 -test with Bonferroni post hoc correction. (C) Comparison of rates of activity in spontaneously active neurons in each group.

Figure 4.

Schematic diagrams of depth of DHNs recorded. Depth for each DHN recording is illustrated on schematic diagrams of L4-5 segment of the spinal cord. Each circle represents the recording depth for an HT neuron (A) and a WDR neuron (B). Open and filled circles represent neurons without SA (SA -) and neurons with SA (SA +), respectively. Open and filled bars represent the mean depth for neurons without SA and neurons with SA, respectively. Note: each circle is randomly arranged on the horizontal axis and does not reflect the medial or lateral position of the neuron in the spinal cord. * $P < 0.05$, SA + vs. SA -, Student's t -test.

Figure 5.

Effect of superficial and deep injection of bupivacaine into the incision on SA of DHNs. Example recordings of neurons with SA from sham (A), skin incision (B), skin plus deep tissue incision (POD1) (C), and skin plus deep tissue incision (POD7) (D) groups. Filled arrows represent time of bupivacaine injections. Bin width = 1 sec. Unit represents each single action potential. (E) Summary of the mean rate of SA before and after bupivacaine injection in each of the 4 groups. Open and filled bars represent before and after bupivacaine injection, respectively. Results are expressed as mean and SEM. * $P < 0.05$, two-way ANOVA followed by paired t -test for comparing the mean rates of SA before and after bupivacaine injection

within each group and one-way ANOVA with Tukey's post hoc test for comparing the mean rates of activity of each group among groups.

Figure 6.

Responses of DHNs to heat stimulation. Example recordings of responses of neurons from sham (A), skin incision (B), skin plus deep tissue incision (POD1) (C), and skin plus deep tissue incision (POD7) (D) to heat stimulation. Inset: Digitized oscilloscope trace of action potentials during heat stimulation. Open arrow head, heat-evoked action potential with large amplitude from a mechanically identified WDR neuron; filled arrow head, heat-evoked small amplitude action potential from another unidentified DHN. Bin width = 1 sec. Unit represents each single action potential. (E) Percentages of neurons responding to heat stimulation in each of the 4 groups. (F) Total action potentials during the heat stimulation. (G) Peak discharge frequencies during the heat stimulation. (H) The temperature thresholds for activation of DHNs

in each of the 4 groups. Results are expressed as mean and SEM. $*P < 0.05$, $***P < 0.001$, one-way ANOVA, Tukey's post hoc test.

Figure 7.

Mechanical responses and mechanical RF of WDR neurons. The most mechanosensitive site of neuron is represented as each dot on the schematic of the hindpaw (A, C). Comparison of peak response frequencies to von Frey filaments of WDR neurons without SA (B) or with SA (D). BL, baseline activity before application of monofilaments. Error bars (SEM) are shown for the sham group and the skin plus deep tissue incision (POD1) group. * $P < 0.05$ sham vs. skin+deep (POD1), § $P < 0.05$ skin vs. skin+deep (POD1), + $P < 0.05$ sham vs. skin+deep (POD7), ++ $P < 0.01$ skin vs. skin+deep (POD7), two-way ANOVA followed by repeated one-way ANOVA measurements with Tukey's post hoc test for the evaluation of effects of groups at each force. (E) Schematic diagrams of RF of WDR neurons from each of the 4 groups measured by a von Frey filament with 240 mN bending force. The gray area depicts the region responsive to the von Frey filament application. The vertical line on the paw represents the location of the incision. (F) The relative sizes of RF areas WDR neurons in each of the 4 groups. The RF areas of skin, skin+deep (POD1) and skin+deep (POD7) group were

normalized to the RF area of the sham group. Results are expressed as mean and SEM. * $P < 0.05$, *** $P < 0.001$, one-way ANOVA, Tukey's post hoc test.