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Reduction of Postincisional Allodynia by Subcutaneous Bupivacaine

Findings with a New Model in the Hairy Skin of the Rat

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Background: **An incision of hairy skin of the rat's back provides a new model for postincisional pain to determine the importance of cutaneous anesthesia.**

Methods: **Male Sprague-Dawley rats were anesthetized with sevoflurane and given a 0.6-ml subcutaneous injection of bupivacaine (0.25%) under the incision site or the medial lumbar dorsum or at the nuchal midline, 30 min before a 1.0-cm skin incision. Mechanical stimuli (von Frey hairs, 18–250 mN) were applied to measure nociception, indicated by twitching of local subcutaneous muscles, the cutaneus trunci muscle reflex. A graded response score, averaging the twitches weighted by their vigor, or a population response score, measuring the fraction of rats that showed any response, was assessed for 3 days before and over 7 days after incision. von Frey hairs were applied 0.5 cm from the incision to test primary hyperalgesia and 2.0 cm contralateral to the incision for secondary hyperalgesia.**

Results: **Incision induced responses to stimuli that had no effect on intact skin (allodynia) and also enhanced responses to forces that normally gave less than the full reflex (hyperalgesia). Hyperalgesia was present 30 min after surgery, peaked at 3–6 h, and persisted through the week; allodynia had a similar onset but was briefer. Both changes were transiently reversed by subcutaneous morphine (2.5 mg/kg intraperitoneal). Subcutaneous bupivacaine (0.25%), injected preoperatively at the incision site and anesthetizing skin for 2–3 h, suppressed primary allodynia for 1 week but had no effect on hyperalgesia. Secondary allodynia was obliterated, and secondary hyperalgesia attenuated by this treatment. Bupivacaine injected subcutaneously at the nuchal midline before surgery was also effective in abbreviating primary and secondary allodynia, with no signs of sedation, ataxia, or preconvulsive behavior.**

Conclusions: **Incision of rat hairy skin changes pain responses, similar to pain in humans. Preincisional subcutaneous bupivacaine selectively suppresses and shortens allodynia for times far outlasting its local anesthesia, an effect largely from systemic actions.**

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PERSISTENT pain after surgical incision presents an obstacle to rapid recovery, often limiting pulmonary function, postural mobility and locomotion, and prolonging hospitalization and bed rest.[1,2] Broader indications suggest that postoperative pain, which for the most part is localized to the site of the incision, predisposes patients to greater morbidity and increased stress that can endure for weeks or longer after surgery.[3,4]

Animal models that have been developed for the study of postincisional pain have focused on changes in threshold for nocifensive responses to mechanical or thermal stimulation, using the glabrous skin on the footpad of the rat.[5,6] Much useful information about alterations of the peripheral nervous system and central nervous system (CNS) after such surgery has been derived from these models. However, most clinical incisions occur on hairy skin, which differs somewhat from glabrous skin in its injury response,[7,8] and successful analgesia of the footpad site of incision promotes weightbearing and direct contact of the incision with supporting surfaces, which in itself may alter wound healing and recovery.

In an attempt to more closely reproduce changes that occur in human surgery, we have developed a new model for postincisional pain on the hairy skin of the back of the rat. A 1-cm long incision through the rat's skin, sparing as much of the underlying fascia as possible and leaving the muscle intact, resulted in hyperalgesia, an increased response to mechanical stimuli that would give only partial nocifensive responses in intact skin, and in allodynia, indicated by nocifensive responses to weak mechanical stimuli that induce no responses in intact skin. Both of these changes occur in most humans after surgical incision (or accidental trauma).[7,9]

In the current study, we sought to test the hypothesis that brief, cutaneous local anesthesia, lasting for approximately 2–4 h in intact skin, could suppress the prolonged postincisional pain. Using 0.5 or 0.25% bupivacaine injected subcutaneously 30 min before the operation to anesthetize an area that circumscribed the intended incision, we found a significant suppression of allodynia without any effects on hyperalgesia, particularly so for secondary responses. Surprisingly, as strong an effect also occurred from the same dose of bupiva-

caine injected many centimeters from the incision, suggesting a systemic action of this drug. These general results are consistent with clinical reports of perioperative analgesia and raise new scientific questions about the different mechanisms or fiber types that subserve these differentially susceptible modes of postincisional pain.

Materials and Methods

These experiments were reviewed and approved by the Harvard Medical School's Animal Care and Use Committee (Boston, Massachusetts). The animals 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.[10]

Handling and Preparation of Animals

Adult male Sprague-Dawley rats (150–160 g at delivery) were kept in plastic cages with soft bedding with access to food and water *ad libitum*, maintained on a 12-h light, 12-h dark cycle. Experiments were conducted in animals that had been handled daily (over 7–10 days) and were thus familiarized with the behavioral tester, the experimental environment, and the specific experimental procedures. Criteria for sufficient handling were an absence of behavioral signs of stress (*e.g.*, immobilization and lack of exploratory behavior in an open field environment, frequent defecation) and, importantly, an extinction of the initially present dorsal cutaneous muscle contractile response to innocuous stroking of the area to be tested, but with a robust, distinctive contractile response to noxious stimulation, *e.g.*, pinprick. This cutaneous trunci muscle reflex (CTMR),[11] which is characterized by movement of the skin over the back produced by reflex twitches of the underlying lateral thoracospinal muscles in response to local dorsal cutaneous stimulation, was studied as a reaction to tactile stimuli using nylon monofilaments of different thickness (von Frey hairs [VFHs]). (Reactions to innocuous stroking and to relatively thin diameter VFHs were intentionally extinguished by repeated handling, as previously mentioned). The hair over the skin to be tested was clipped with electric shears 24 h before testing; local irritation produced by this clipping resolves overnight.

Behavioral Response Evaluation

Testing Responsiveness to Mechanical Stimulation. Each VFH was calibrated on a top-loading electronic balance, using the average of five measurements, made before and after each experiment to ensure that the stimulus intensities remained unchanged. The responsiveness to varying stiffness (forces) of VFHs was used to gauge the sensitivity of each rat's skin, and differences in the measures of such responsiveness were quantitated as measures of postincisional hyperesthesia and thus also as the measure of drug actions. Specifically, each VFH was applied four times in any one trial done at a specified time, each poke was spaced 2–3 s apart, and sequential VFHs were applied in ascending order of stiffness. The recorded responses during each trial were analyzed in two ways. In the graded response analysis, each contraction was scored by the experimenter as 1, 0.5, or 0 based on its vigor, *i.e.*, speed and rostrocaudal extent; a single robust contraction, such as occurred from pinprick (5–7 cm of the skin contracts, along the rostrocaudal axis), was scored as 1.0, a weaker or shorter contraction (2–4 cm or less) was scored as 0.5, and no visible contraction was scored as 0. Because four stimuli were applied for each VFH, the maximum response that could be scored was 4.0, and this could be decreased by units of 0.5 until there was no response. These raw scores were normalized by the maximum possible response (= 4.0), and the resulting scores given as a weighted average, the graded response, ranging from 0 to 1, calculated as follows:

$$\text{Graded Response} = \Sigma\, v_i/4,$$

where v_i is the vigor of the CTMR ($0 \rightarrow 1.0$) to the *i*th stimulation and the sum is taken over all four stimuli for each VFH. This is not based on a truly continuous scale, nor is it proportional to intensity of pain, but rather multiplies the probability of response with the weighted motor (nocifensive) response.

In the population response analysis, any detectable contraction during VFH application was scored as 1, and an absence of contraction was scored as 0, for any of the four pokes from a single VFH given in one trial. This number is summed for all animals in a treatment group and divided by the number of animals. The population response thus equals the fraction of animals that respond to punctate mechanical stimulation.

Baseline responsiveness was determined for 3 consecutive days in all animals before administration of drug, administration of vehicle, or incision. It was statistically constant over this time, neither varying after the end of the handling period nor changing after the rats had recovered ($t > 20$ min) from a single episode of sevoflurane anesthesia (data not shown).

Although this visual assessment of the strength and extent of muscle contraction for the graded response is only semiquantitative, and the scoring of extent of contraction is dependent on learned judgment, we limited the subjective bias by having only one investigator familiarize by handling and subsequently test any one cohort of rats (usually numbering eight) for all the different procedures done in that cohort; the scored results among the individual rats in one cohort were relatively consistent, with coefficients of variation of 20% or less (see Results section). In addition, the effects of incision

on cutaneous hyperalgesia in control, vehicle-injected rats were compared between two different investigators (A. M. D. and E. R.) on two separate cohorts of rats tested 5 months apart, with statistically identical results. Furthermore, the scoring of the population responses, requiring no judgment of contraction vigor, was effectively identical among investigators. Thus was the reproducibility of graded postincisional hyperalgesia and allodynia established in this model.

Assessing Postincisional Hyperalgesia. Using these procedures, we examined the changes in responsiveness to VFHs of different forces on the area of the skin adjacent to an incision injury (primary hyperalgesia; at 0.5 and 1 cm) and at 2 cm from the incision (secondary hyperalgesia). Previous publications trace the anatomy of this innervation, showing that peripheral innervation of the skin (and the contained muscles) is unilateral such that changes measured contralateral to an injury site cannot be due to injury of nerves in the tested region.[11]

Before any procedure, the dorsolumbar region of the sheared rat's back was divided into four quadrants with a permanent marker. The location where an incision would be made was examined with VFHs to establish the immediate preoperative responsiveness and to compare it to the baseline values from the preceding 3 days. After the incision, which was made during general anesthesia (see Surgery and Drug Delivery), cutaneous testing was resumed with von Frey filaments applied at 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 8 h, 24 h, and once a day for 15 days. Any nonzero response to 18 mN, an ineffective force before an incision, indicated tactile *allodynia*. The increased responses to stronger forces that had been partially effective in preincision skin are here termed *hyperalgesia*. We confirmed that these changes, here collectively termed *hyperesthesia*, were evidence for pain perception by noting that the increased responses were transiently suppressed by systemic morphine (see Results, Effect of Morphine), analogous to the presence of CTMR in swine in response to noxious heating being suppressed by systemic opiates.[12] A similar increase in local response of muscles to tactile stimulation in human neonates and infants after surgery has also been interpreted as an index of increased pain.[9] In the current studies, the initial handling of the rats led to an extinction of the CTMR to nonnoxious tactile stimuli, and this, taken together with the aforementioned observations, justifies the interpretation of the increased skin responses as heightened pain.

Although responses were always measured at distances of 0.5, 1, and 2 cm ipsilateral and 2 cm contralateral from the incision, the results measured at 0.5 cm (primary hyperesthesia) and at 2 cm contralateral (secondary hyperesthesia) are emphasized here. The overall pattern of change was the same at each distance, although the amplitude of the enhanced responsiveness decreased with distance from the wound. The area under the curve for individual forces, integrated over postincisional time, was taken as an overall measure of the degree and duration of postincisional hyperalgesia.

Surgery and Drug Delivery

The experimenter in these studies was not blinded to the drug used for injection, because any cutaneous anesthetic effects of the local injection of bupivacaine would be immediately apparent to the observer.

All rats were anesthetized twice with sevoflurane, delivered *via* nose cone from saturated gauze placed in the bottom of a small (50-ml) beaker. During the first anesthetic period, the sites of drug injection, of incision, and of loci at 0.5, 1, and 2 cm distance from the incision were marked on the back using a transparent plastic template (fig. 1). Each rat received a 0.6-ml-volume subcutaneous injection (bupivacaine or vehicle) in the back, at the demarcated site, delivered from a 1-ml tuberculin syringe through a 25-gauge, 16-mm needle (Becton Dickinson and Co., Franklin Lakes, NJ), inserted to its full length. This volume spread sufficiently to raise a bubble under the skin of approximately 1.2 cm in diameter, within which the incision was later made. Bupivacaine or control vehicle (0.15 M NaCl buffered with 0.0 1M PIPES, pH adjusted to 6.8) was injected subcutaneously 30 min before the incision to allow the active agent to penetrate into the epidermis. Pilot studies showed that this timing would give full block by the time of the incision. The animals were then placed in separate cages to recover from the general anesthetic (taking < 2 min) and for the subcutaneous substance to spread and penetrate. After 30 min, sevoflurane anesthesia was administered again, and at this time, a 1.0-cm longitudinal (parallel to the midline) incision of the skin (trying to avoid the fascia) was made in a sterile manner (skin washed with povidone-iodine solution; Clinidine®; Clinipad Corp., Rocky Hill, CT; instruments previously autoclaved, sterile surgical blade No. 10). (In some experiments, the incision was irrigated with 0.5 ml or so of the same solution used in the preincision injection, but this did not alter the postincisional analgesia [data not shown], and the practice was not applied further.) The wound was closed with one suture of 3-0 or 4-0 silk (Look®; Surgical Specialties Corp, Reading, PA), and the rats were returned to their separate cages to recover from anesthesia before testing began, 30 min later.

Morphine Delivery

In experiments using no other drugs, morphine was injected (2.5 mg/kg intraperitoneal) 4 h after the incision, and the CTMR to allodynic-level and to hyperalgesic-level stimuli was followed for the next 4 h and at 24 and 48 h after incision.

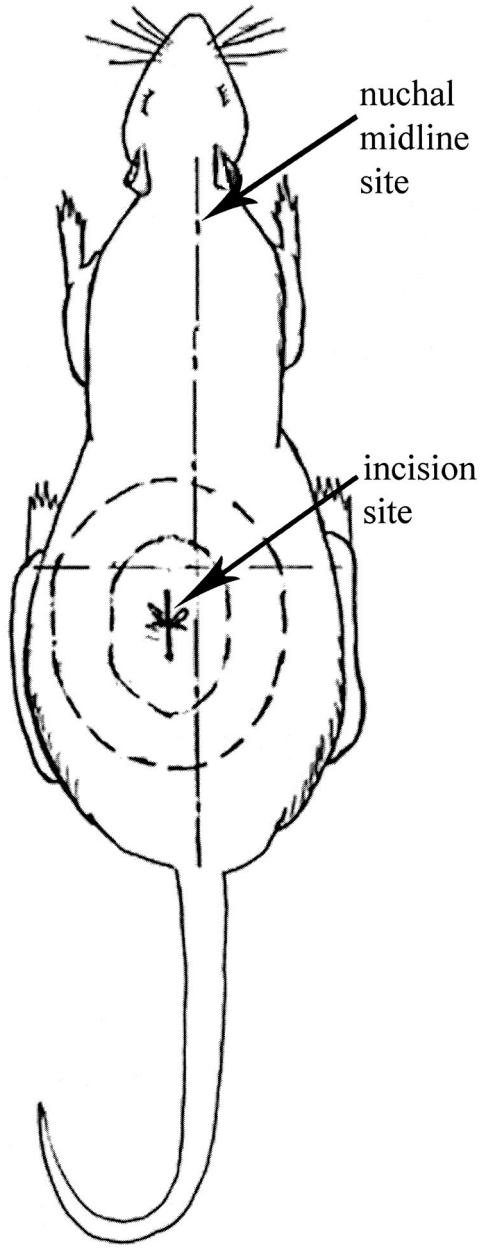

Fig. 1. Location of the 1-cm-long skin incision with single suture, posterior to the L4 transverse process and 0.5 cm from the midline. The two injection sites for subcutaneous bupivacaine are shown by *arrows*. The *broken-line oblongs* are drawn on the skin at 1 and 2 cm distance with the same template that marks the incision line, all while the rat is under sevoflurane anesthesia.

Drugs

Bupivacaine solutions (0.25 and 0.5%) were made from the hydrochloride salt of the racemic drug (Sigma-Aldrich, St. Louis, MO; also the supplier of PIPES buffer) by dissolving in PIPES-buffered saline (0.15 M NaCl), the final pH being set at 6.8 at 20°–22°C. Morphine sulfate was from Baxter Healthcare Corp. (Deerfield, IL) and was dissolved to a stock concentration of 1 mg/ml in phosphate-buffered saline, pH adjusted to 7.4. Sevoflurane (Ultrane®; Abbott Laboratories, North Chicago, IL) was poured directly onto a gauze pad in a beaker. A scavenging system with a funnel placed above the rat's head and attached to a vacuum line minimized stray sevoflurane vapors.

Data Analysis and Statistics

Postincisional hyperalgesia or allodynia, with no drug treatment, was determined by comparison of responses between rats that received a vehicle injection followed by incision with those that were injected with vehicle but received no incision. Values are graphed as mean ± SD, with the latter to show the spread of data, although a normal distribution was not assumed. Comparisons between graded responses in intact skin and those after incision (both after vehicle injection) and between vehicle- and bupivacaine-treated groups after identical incisions were made in each group, using nonparametric statistics (Mann–Whitney U test), to compare each separate time point and each particular force of VFH. Importantly, statistical analyses only compared responses for the same VFHs and for the same times, preoperatively and postoperatively, such that no corrections for repeated measures or for multiforce measurements were required. The same comparisons between these respective groups' population responses were made with the chi-square test (Statview software; Cary, NC). Statistical significance was set at $P < 0.05$.

Results

Preincision Controls, Intact Skin

Stimulation of the intact skin by von Frey hairs using the identical protocol of timing and order of forces used for testing postincisional pain (see Materials and Methods section) resulted in a transient decrease in graded responsiveness (fig. 2A). At the low frequency of testing, once per day, that preceded the time of incision (t = 0), the responsiveness was constant, but just after t = 0, when the tests were applied every 30 min for several hours (see Materials and Methods section), the responses to the three intermediate forces (36, 90, and 143 mN) decreased, although the response to a very stiff VFH, 520 mN, remained vigorous. When the testing frequency returned to the lower values of 1–0.25 per hour, responsiveness gradually returned to the initial baseline value and remained there for 1 week of testing (data not shown).

Injection of vehicle subcutaneously, below the testing site, induced a transient, albeit mostly insignificant hyperresponsiveness (fig. 2B). These minor, vehicle-induced changes dissipated to baseline by 8 h after the injection and also remained at this pretreatment level for at least 1 week.

Bupivacaine Analgesia in Intact Skin

Responses in skin were totally abolished for 90–120 min by subcutaneous injection of 0.6 ml bupivacaine, 0.25%. Complete blockade at the injection site was

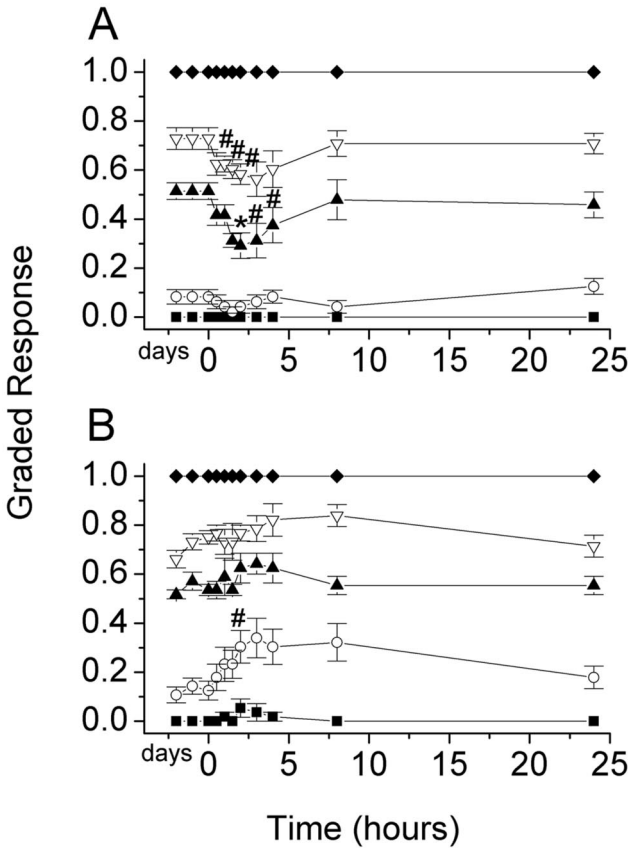

Fig. 2. Graded responses to a range of von Frey hairs, shown as mean ± SD. (*A*) Applied to intact, uninjected skin with the same pattern and frequency as used to test for postincisional pain (n = 6). A significant stimulus-dependent hypoalgesia occurs during frequent testing by intermediate-sized von Frey hairs. (*B*) Applied to skin after injection of vehicle (n = 7). # $P < 0.05$ *versus* baseline of responses that were tested daily on the preceding 3 days, indicated by the points graphed in negative time. Responses were significantly greater than baseline values only during the period of frequent testing (0–5 h) and had returned to baseline by 24 h. von Frey hair forces were 18 (■), 36 (○), 90 (▲), 143 (▽), and 520 mN (♦).

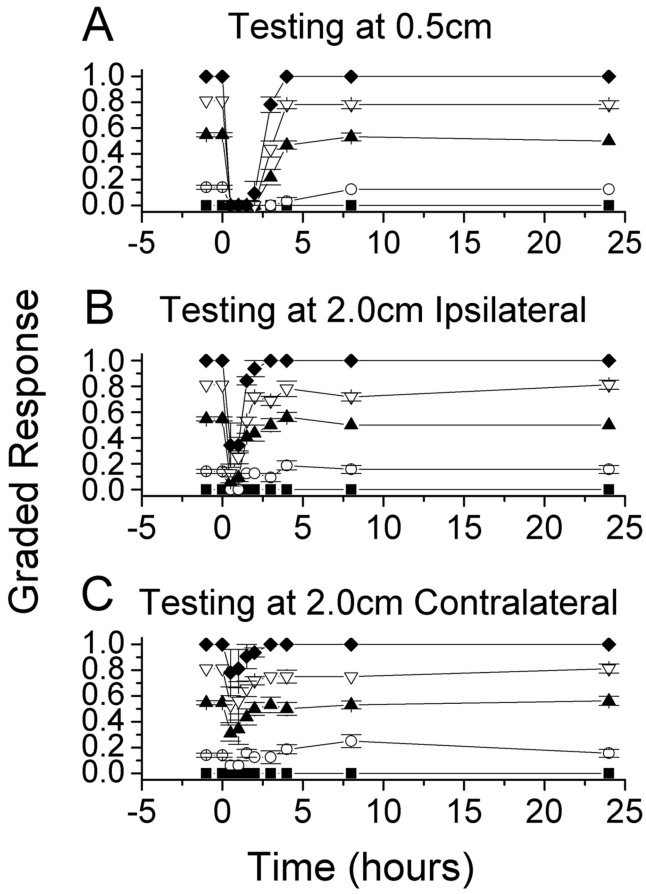

Fig. 3. Anesthesia of intact skin by 0.25% bupivacaine (0.6 ml). Normalized responses, shown as means ± SD, to a range of von Frey hairs measured at 0.5 cm from the injection site (*A*), 2 cm ipsilateral from injection site (*B*), and 2 cm contralateral from the injection site (*C*) (n = 4). von Frey hair forces were 18 (■), 40 (○), 90 (▲), 150 (▽), and 520 mN (♦).

reached by 10 min after the injection (a time not usually tested here), and partial recovery was evident at 3 h after the injection, with a return to baseline sensitivity by 4 h after injection (fig. 3A). When measured at 2 cm from the injection site either on the ipsilateral (fig. 3B) or contralateral side (fig. 3C), the analgesia was weaker and briefer. Analgesia from subcutaneous 0.5% bupivacaine showed an almost identical profile (data not shown). Skin responses after bupivacaine, as after vehicle, remained at their preinjection, baseline levels for 7 days after the regression of local anesthesia.

No adverse systemic reactions, such as sedation, ataxia, or preconvulsive twitches, followed any bupivacaine injections, and there were no signs of local inflammation or irritation.

Postincisional Pain Responses

Responses in Unanesthetized Skin. Skin incision caused rapidly increasing, long-lasting increases in responses to cutaneous tactile stimulation. The primary graded responses, to stimulation 0.5 cm from the wound, with only vehicle injected before the incision, were increased by 30 min, the earliest time of testing after incision (fig. 4A), and remained increased for at least 1 full week (fig. 4B). (Throughout this report, the responses in treated skin to any one VFH at any specific perioperative time are compared only to the responses in untreated skin to the same VFH at the same time, *e.g.*, incision with vehicle *vs.* no incision with vehicle or incision with vehicle *vs.* incision with bupivacaine.) Maximum hyperalgesia and allodynia for the graded responses were reached by 3–4 h after surgery (fig. 4A) and changed only slowly afterward; allodynia (signaled by the response to 18 mN) decreased faster than hyperalgesia (signaled by responses to 40 or 90 mN; fig. 4B), although both were significantly greater than preincisional baseline or vehicle control values (compare fig. 2B) for the entire postincisional week. (In four animals, CTMRs to forces as low as 5.9 mN were induced at 6–24 h after incision, although no response occurred to stimulation with a soft-bristle watercolor paintbrush [No. 10,

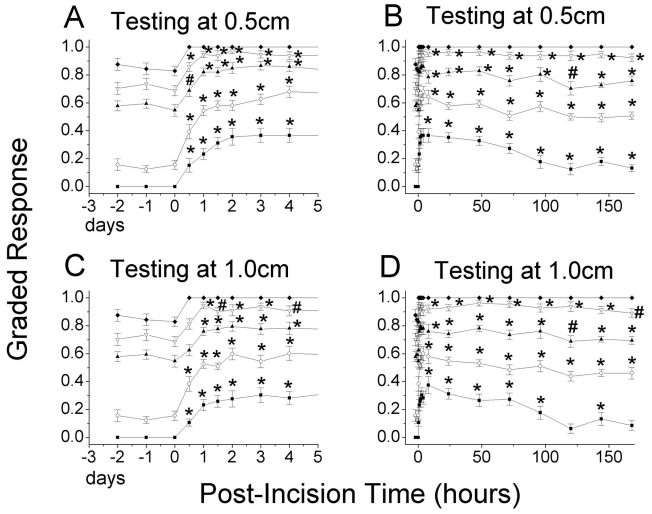

Fig. 4. The time course of graded primary allodynia and hyperalgesia (as mean ± SD) after incision, showing the kinetics of development for the first 4 h (*A* and *C*) and over 7 days (*B* and *D*), at 0.5 and 1.0 cm from the incision site, preceded by vehicle injected subcutaneously at the incision site. Statistical significance of the response was determined by comparison to the corresponding time point for that force in vehicle-injected rats with no incision (*e.g.*, fig. 2B) by Mann–Whitney U test: * $P < 0.005$, # $P < 0.05$ (n = 16). Three days of preincision baseline values are shown by the points for −2 to 0 days. The forces used were 18 (■), 40 (○), 90 (▲), 150 (▽), and 250 mN (♦).

all sable; Windsor and Newton, London, England]. No other experiments to quantitate mechanical thresholds were conducted.)

Measured at 1.0 cm from the wound, changes in graded response were very similar to those at 0.5 cm, (figs. 4C and D). By comparison, at 2.0 cm from the wound, whether on the ipsilateral (figs. 5A and B) or the contralateral side (figs. 5C and D), allodynia was smaller,

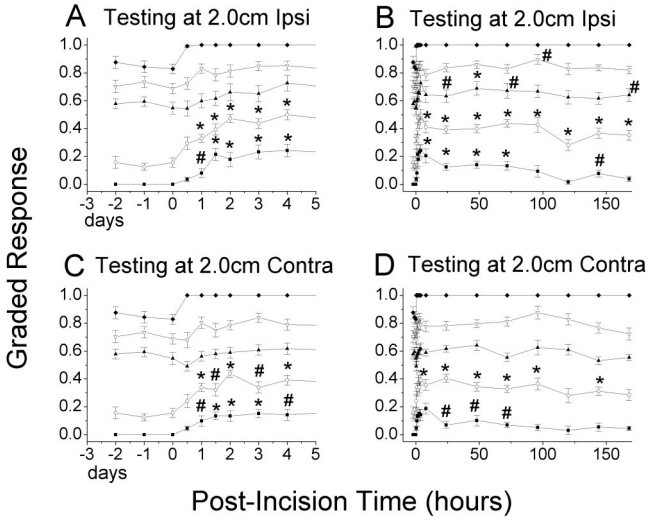

Fig. 5. Time course of graded secondary allodynia and hyperalgesia (as mean ± SD) measured at 2 cm ipsilateral (*A* and *B*) and 2 cm contralateral (*C* and *D*) from the site of the incision, after subcutaneous vehicle. The early changes in response are shown in *A* and *C*; the changes over 1 week are shown in *B* and *D*. Significance cues and force-denoting symbols (*, #) are the same as in figure 4 (n = 16).

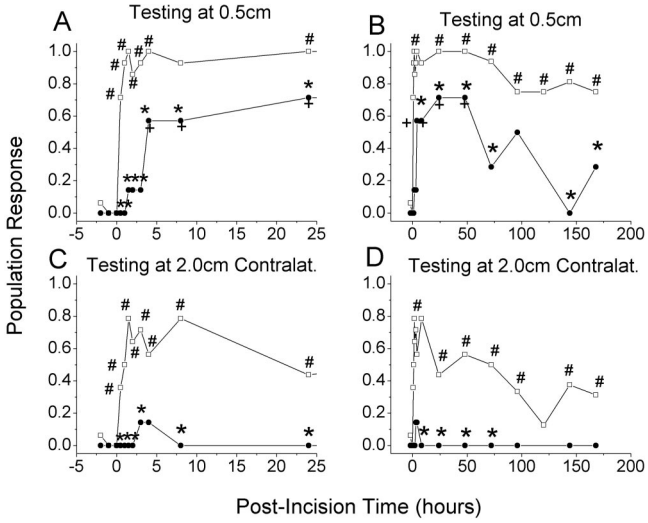

Fig. 6. The population response for allodynia, judged by any detectable cutaneous trunci muscle reflex to 18-mN stimulation, and equal to the fraction of animals that respond to stimulation, of all those tested. Primary responses for the first day (*A*) and an entire week (*B*) and secondary responses (*C* and *D*) for the same respective periods. □ = vehicle (n = 16); ● = 0.25% bupivacaine (n = 8). $P < 0.05$, # incision plus vehicle *versus* baseline, + incision plus bupivacaine *versus* baseline, * incision plus vehicle *versus* incision plus bupivacaine.

took twice as long to reach significance above vehicle only, and resolved faster than at the nearer sites (compare with figs. 4B and D). The hyperalgesia in response to 40 mN was also smaller and briefer, and no significant hyperalgesia to forces above 40 mN developed. Therefore, secondary graded responses to the incision were less severe than primary changes, and the area of skin directly responsive to local injury and inflammation, accounting for the primary response, seemed to be contained within a 1- to 2-cm diameter region around the incision.

Effect of Morphine. In four rats untreated with any other drugs, systemic morphine (2.5 mg/kg intraperitoneal) was delivered at 4 h after incision. Both graded allodynia and hyperalgesia were transiently reversed by approximately 80% of the way back to their baseline responses, an effect that peaked at 30 min after the morphine and had dissipated by 4 h later (data not shown). No ataxia or sedation was apparent at this morphine dose, confirming that a specific analgesic could suppress the increased CTMR and qualifying it as a bona fide measure of pain.

Population Responses to Incision. To remove any subjective distortion of the results due to different assessments of contraction vigor between observers, and to separate the likelihood of response from the degree of contraction, we also analyzed the data by an all-or-none score, the population response (see Materials and Methods section). Importantly, even in the baseline condition, most of the rats responded at least once in a trial to VFHs of 40 mN or greater, and all responded to these forces after incision (giving the maximum population

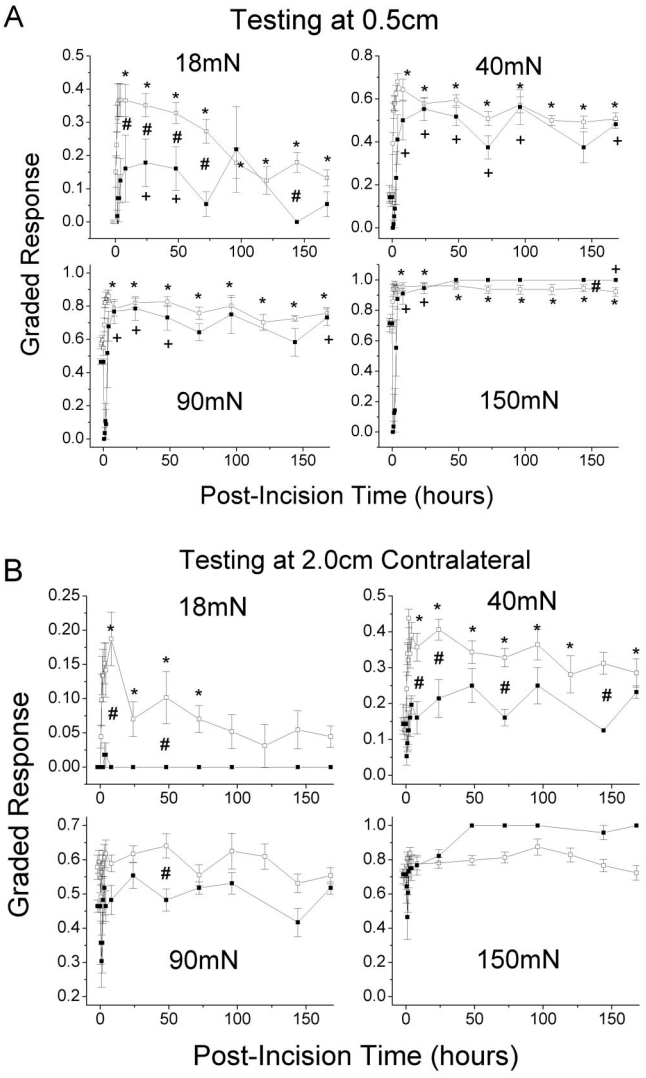

Fig. 7. Graded responses to four forces of von Frey hairs in rats preinjected with vehicle (□, n = 16; data from fig. 4) or with 0.25% bupivacaine (■, n = 8) at the injection site. Peak changes in response (regardless of when they occur) and area under the curve for response above vehicle controls (no incision) over the 7 days after incision are collected in table 1. Responses to von Frey hairs applied at 0.5 cm (*A*) and 2 cm (*B*) contralateral from the incision show the primary and secondary changes, respectively. Data are shown as mean ± SD. Statistics compare responses in bupivacaine- *versus* vehicle-treated rats, both of which have incision (# $P < 0.05$) and also compare each of these groups with the ones that receive vehicle but have no incision (data not shown here; see fig. 2B). *,+ $P < 0.05$, respectively, for these two groups' comparisons.

response score of 1.0; data not shown), but none responded to the 18-mN VFH at any time beyond 5 h after vehicle injection (data not shown). The population response analysis is therefore restricted to allodynia, here assayed by responses to 18-mN VFHs. The time course of the population response to incision differs from that of the graded response in two respects. First, it increases faster. Within 1 h of the incision, all the rats were responsive to 18-mN stimulation applied at 0.5 cm (figs. 6A and B, open squares), whereas in these same rats, the primary graded response to 18 mN at this location had only reached approximately half of its peak value at that time (fig. 4A, filled squares). At the time of maximum graded response at 0.5 cm (3–8 h), all of the rats were allodynic, but they responded on average with only 0.4 of a fully vigorous twitch. An analogous relation was observed for secondary allodynia, where testing at 2 cm contralateral invoked very weak graded responses (fig. 5C) but one detected in 80% of the rats (fig. 6C); the population response peaked at 1.5 h, approximately the same time when the maximum graded allodynic response occurred.

Table 1. Comparison of Bupivacaine and Vehicle in the Back on Postincisional Primary Allodynia*

	Peak Allodynia†	Duration of Allodynia‡, h	Area under the Curve
Vehicle (n = 16)	0.48 (0.25–0.75)	168 (1 week)	41.8 (8.8–64.7)
Bupivacaine (n = 8)	0.25 (0–0.75)§	48§	18.7 (0–51.2)§

* Testing 0.5 cm from incision with 18-mN von Frey hairs, graded response, with range in parentheses, after 0.25% bupivacaine. † Maximum graded response regardless of time of occurrence. ‡ Time over which allodynia is significantly greater than baseline. § Significant difference between vehicle and bupivacaine.

A second difference between these two types of analysis was in the persistence of the response. The graded assessment of primary allodynia decreased by more than half of its peak value at 1 week (168 h) after the incision (fig. 4D), during which time the population responding decreased by only approximately 20% (fig. 6B). The graded response for secondary allodynia decreased to insignificance after 72 h (fig. 5D), whereas the population response for secondary allodynia was significant for most of the entire week (fig. 6D).

Actions of Bupivacaine on Postincisional Pain

Effects of Bupivacaine on Primary Postincisional Pain. Local subcutaneous administration of bupivacaine selectively suppressed postincisional allodynia. When the local anesthetic was injected at the incision site 30 min before surgery, at concentrations of either 0.25 or 0.5% (data not shown), the primary graded allodynia (response to 18 mN) was significantly reduced (fig. 7A). Early peak primary allodynia was 48 ± 19% of control (0.48 with vehicle *vs.* 0.25 with bupivacaine (0.25%); table 1) and was significantly suppressed from vehicle-treated incisional values at each time point up to 72 h (fig. 7A; the values in table 1 are the means of the maximum responses from each animal regardless of their time of occurrence, accounting for the difference from the maximum values in the figures). The area under the curve for graded allodynia, integrated over the 7 days after incision, was similarly reduced to 45% of control (42 h for vehicle and 19 h for bupivacaine; table 1).

Table 2. Comparison of Bupivacaine and Vehicle on Postincisional Secondary Allodynia*

	Peak Allodynia	Duration of Allodynia†, h	Area under the Curve
Vehicle	0.30 (0.13–0.63)	72	12.38 (1.75–24.4)
Bupivacaine	0.02 (0–0.13)‡	0	0.06 (0–0.44)‡

Mann–Whitney results for areas under the curve: vehicle *vs.* bupivacaine: $P < 0.001$. Mann–Whitney results for peak allodynia: vehicle *vs.* bupivacaine: $P < 0.001$.

* Testing 2.0 cm from incision on contralateral side with 18-mN von Frey hairs, mean graded response, with range in parentheses, after 0.25% bupivacaine. † Time over which allodynia is significantly greater than baseline. ‡ Significant difference between vehicle and bupivacaine.

When assayed as the population response, allodynia at 0.5 cm was also delayed and its maximum value was reduced by bupivacaine; 30% fewer rats responded to 18-mN stimulation at 4 h after incision when given bupivacaine, a time when local anesthesia had fully regressed (fig. 6A). The primary population response of allodynia was also shortened by bupivacaine, losing significance above baseline beyond 48 h after surgery, in contrast to the week-long allodynia in vehicle-treated rats (fig. 6B).

In contrast to these allodynic differences, primary hyperalgesia at 0.5 cm, registered by responses to the stronger forces, was not modified by bupivacaine. Early peak graded responses to 40–150 mN were the same in vehicle-treated and bupivacaine-treated animals, as were the areas under the curve for the week after incision (fig. 7A).

Actions of Bupivacaine on Secondary Allodynia and Hyperalgesia. Bupivacaine profoundly reduced secondary allodynia from an incision. As reported above, postoperative changes in mechanical sensitivity at regions of skin 2 cm from the incision were smaller and briefer than those at the incision site. On the contralateral side, in particular, graded allodynia peaked at 60% of the respective amplitude measured at the ipsilateral site (compare tables 1 and 2) and remained significantly above baseline for only 72 h compared with the full 7 days at the incision (fig. 4B *vs.* 5D). Secondary hyperalgesic responses to 40 mN were similarly smaller and briefer, and no secondary hyperalgesia registered in response to stronger VFHs (fig. 5D).

Subcutaneous bupivacaine injected at the incision site strongly suppressed secondary responses (table 2). No allodynia developed at 2 cm contralateral to the incision (fig. 7B) because there was no significant population response at any time at this distance (figs. 6C and D, filled circles), and, interestingly, unlike the primary response elicited at 0.5 cm (fig. 7A), peak secondary hyperalgesia tested by 40-mN stimulation was attenuated by approximately half at all times by bupivacaine (fig. 7B). None of the responses to 40-mN stimulation at 2 cm contralateral in bupivacaine-treated rats were significantly increased over those in the preincision baseline (or the vehicle only–treated) rats. Therefore, the inhibition by bupivacaine of secondary responses, measured at a distance, exceeds inhibition of primary responses measured at the incision site, also the location of injection of bupivacaine, and where the acute anesthetic actions of bupivacaine are stronger and longer lasting (fig. 3).

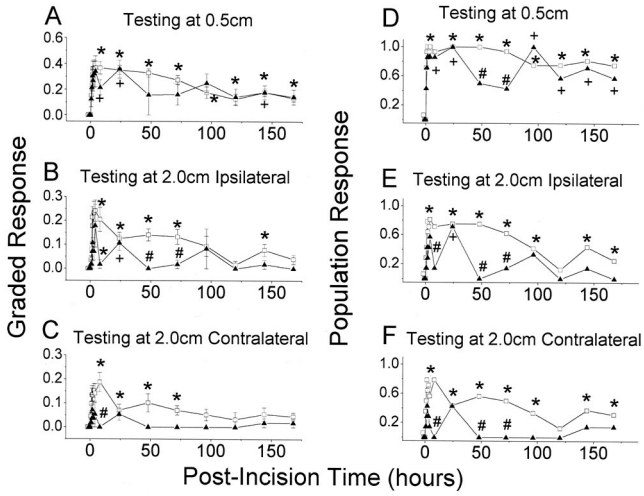

Fig. 8. Graded responses (*A–C*) in rats injected with vehicle (□) at the incision site (n = 16, as in fig. 4B) or with 0.25% bupivacaine, 0.6 ml (▲), at the nuchal midline (n = 7), probed with an 18-mN von Frey hair applied 0.5 cm (*A*) or 2 cm (*B*) from the incision on the ipsilateral side or 2 cm from the incision on the contralateral side (*C*). Population responses (*D–F*) for allodynia tested by 18-mN stimulation after injection of vehicle (□; n = 16) or 0.25% bupivacaine at the nuchal midline (▲; n = 8). Primary allodynia is reported by stimuli applied at 0.5 cm (*A*) and secondary allodynia by those at 2 cm (*B* and *C*) from the incision site. Data shown are mean ± SD. Significance from Mann–Whitney U test shows $P < 0.05$, # for rats receiving incision treated with bupivacaine *versus* those treated with vehicle; * for rats receiving incision treated with bupivacaine *versus* vehicle-treated rats with no incision (data not shown here; see fig. 2B and text).

Effects of Subcutaneous Bupivacaine Injected at a Distant Site. To account for the possibility that systemic bupivacaine might contribute to the suppression of allodynia, we conducted separate experiments in which rats were given the same dose (0.6 ml) of 0.25% bupivacaine but were injected at the nuchal midline 30 min before the incision (compare fig. 1). (Injection of vehicle at this site in four rats before incision on the back resulted in changes in peak graded responses, time of peak, and area under the curve that were indistinguishable from those in rats with vehicle injected at the incision site, *i.e.*, fig. 4; data not shown.) With the exception of the immediate local anesthetic suppression of all responses for incision-site bupivacaine, almost certainly due to local cutaneous anesthesia, the suppression of primary graded allodynia from the nuchal midline injection of bupivacaine resembled that from injection of bupivacaine at the incision site (fig. 8A). Both primary and secondary graded allodynia were reduced in degree and duration (figs. 8A–C and table 3). Although the initial increase of

Table 3. Effects of Nuchal Midline Injected Bupivacaine on Primary and Secondary Postincisional Allodynia

	Peak Allodynia	Duration of Allodynia‡, h	Area under the Curve
Primary allodynia*			
Vehicle	0.48 (0.25–0.75)	168 (1 week)	41.85 (8.8–64.7)
Bupivacaine	0.5 (0.25–0.88)§‖	144	33.4 (5.4–74.8)§‖
Secondary allodynia†			
Vehicle	0.30 (0.13–0.63)	72	12.38 (1.75–24.4)§
Bupivacaine	0.16 (0–0.38)§‖	0	2.39 (0–8.9)§

* Testing 0.5 cm from incision with 18-mN von Frey hairs, mean graded response, with range in parentheses. † Testing 2.0 cm from incision on contralateral side with 18-mN von Frey hairs, mean graded response, with range in parentheses. ‡ Time over which allodynia is significantly greater than baseline. § Significant difference from vehicle. ‖ Significant difference from bupivacaine (0.25%) injected at site of incision.

primary allodynia (0–4 h) was unaffected by this treatment (fig. 8A), this same phase of secondary allodynia was noticeably blunted (fig. 8C). Postincisional hyperalgesia was unaltered by systemic bupivacaine (data not shown), as had been the case for bupivacaine injected at the incision site.

Analysis of the population response showed a similar pattern, with significant suppression of primary allodynia at 48 and 72 h (fig. 8D), a parallel reduction measured at 2 cm ipsilateral (fig. 8E), and near total abolition of the secondary population response at 2 cm contralateral (fig. 8F). The inhibitory effects of bupivacaine were absent for the first few hours of the primary population response but occurred at earlier postincisional times for the secondary responses (figs. 8D and F).

The suppression of the immediate postincisional population response was greater when bupivacaine was injected at the incision than at the nuchal midline (compare fig. 6C *vs.* fig. 8F, 78% *vs.* 44%; $P < 0.02$, chi-square), showing that this rapid phase of secondary allodynia is driven strongly by the activity arising from the incision site. Because tactile anesthesia from subcutaneous injections of intact skin on the lumbar back did not extend beyond 3 cm (fig. 3C) and the nuchal midline injection was 8.3 ± 0.1 cm (n = 4) from the middle of the incision, it seems that the distant source of prolonged inhibition of allodynia produces a systemic level of bupivacaine that probably acts predominantly on the CNS.

Discussion

A major goal of the current study was to develop a clinically relevant model for incident pain after an incision in hairy skin. We documented allodynia and hyperalgesia that were quantifiable, spatially separable as primary and secondary, and highly reproducible between different investigators in our laboratory, both in degree and in time course, thus minimizing concerns about the semiquantitative nature of the graded CTMR assay and its potential subjective contamination. Furthermore, in the experiments where bupivacaine was administered systemically by distant subcutaneous injection, the time course of appearance of primary allodynia was unchanged, although the later phases were suppressed, implying that there may be different mechanisms for an induction and for a maintenance phase of postincisional pain. Importantly, because the overall analysis provides an indication of stronger responses to increasingly intense stimuli, it allows the possibility of separating allodynia from hyperalgesia, which others have intimated correspond to different cutaneous fiber types and, possibly, to different mechanisms of sensitization.[13,14] In this way, and because it examines hyperesthesia in hairy skin, it differs from the most popular current animal model for postincisional pain, one that uses the glabrous skin of the paw.[5]

Our observation of a selective effect of bupivacaine on allodynia, without modifying hyperalgesia, also supports the existence of different anatomic pathways and pathologic mechanisms for these two phenomena, as elaborated below (see Which Sensory Neurons Are Involved in Postincisional Pain Changes?). In addition, the nearly identical inhibition of secondary allodynia by bupivacaine injected locally or at a distance from the incision supports a systemic antiallodynic action of this drug and is consistent with a central mechanism for the persistent suppression of postincisional allodynia by this local anesthetic.[15–17]

Although the doses of bupivacaine that we used, 1.5 or 3.0 mg, corresponding to 7.5 or 15 mg/kg rat body weight, would be in the toxic range both for rats (8–12 mg/kg[18]) and for humans (1.6–3.0 mg/kg for CNS and cardiovascular system effects, respectively[19]) if the drug was delivered intravenously, subcutaneous delivery results in a much slower release of drug into the systemic circulation[20] and much lower peak plasma concentrations. For example, lidocaine administered subcutaneously to mice produces a 13-fold lower peak plasma concentration than if administered intravenously.[21,22] None of the rats studied here ever showed any of the well-recognized tremors indicating CNS toxicity or the hemodynamic reactions to cardiovascular toxicity[23,24] and were not apparently intoxicated.

Characterizing Changes in CTMR from the Incision

The behavioral response used in this new model, the cutaneus trunci muscle reflex, is a localized reflex contraction of rostrocaudally aligned muscles underlying the site of sensory stimulation.[11] The familiarization of rats with the experimenter and the procedures during the initial handling period is critical for the success and specificity of this model. When the young (4- to 5-week-old) rats are first received, they are anxious, curious, and

highly responsive to all tactile stimulation; even the lightest stroking with a blunt stimulus or the thinnest VFH on the back triggers these local contractions. However, after 2–3 days of 5- to 10-min-long handling sessions, these local responses to nonnoxious stimuli have been accommodated, but the reactions to frankly noxious stimuli, *e.g.*, pinprick, remain after handling, providing a specific and clearly visible nocifensive response in unrestrained rats. The transient reversal of allodynia and hyperalgesia at the wound site by systemic morphine further testifies to the pain-reporting nature of these responses.

Other studies have used the CTMR to investigate experimental pain, but the noxious stimuli in animals were usually pinching or strong pinprick,[11,25,26] where an all-or-nothing response indicated some level of nociceptive activation in intact skin. Andrews and Fitzgerald[9] applied VFHs to the trunk of neonates or infants before and after surgery and found a postoperative reduction in the force near the wound required to stimulate local contraction of abdominal muscles, in agreement with the report presented here. We used graded punctate mechanical stimuli at different distances from the incision to elicit responses that could be scored either by an average of weighted contractions (the graded response) or by an all-or-none score (the population response). These procedures allowed us to separate allodynia from hyperalgesia and to discriminate primary from secondary hyperalgesia in hairy skin that was relatively uniform over the incision and test sites.

By testing at different distances from the incision, we could separate primary allodynia that remained significant for at least the week of testing (and for up to 3 weeks in preliminary experiments not reported here) from secondary allodynia, measured at 2 cm contralateral, that was smaller in degree and of shorter duration and, strikingly, almost totally abolished by bupivacaine. Comparison of the profiles of graded responses and population responses in untreated rats showed first that the initial fraction of animals with primary or secondary allodynia was not very different (1.0 or 0.8, respectively), indicating that most of the difference in graded response was due to a less vigorous contraction, and second, that the fraction of responders decreased only slightly during the week after the incision, whereas the vigor of these responses faded quite substantially.

Secondary hyperalgesia (and allodynia) in experimental animals and humans, in response to thermal or chemical injury, for example, has been shown to result from sensitization of neurons in the CNS.[13–17,27,28] The neuroanatomy of the CTMR reveals that the afferent fibers converge to the spinal cord from unilateral sites and that activation of muscles on the contralateral side requires afferent processing through the spinal cord.[11] Therefore, the increase of CTMR responses to stimulation contralateral to the incision requires changes in the CNS, and its strong, prolonged suppression by bupivacaine supports the conclusion that this effect is largely through systemic rather than local actions. The plasma lifetime of bupivacaine in rats is less than 6 h,[20] so the week-long effects are the consequence of actions that occur in the perioperative period, when afferent discharge from injured nerves is most robust[29–31] and acute spinal sensitization is first induced.[32] Interestingly, the initial increase in primary allodynia is unaffected by systemic bupivacaine, but persistent allodynia is abbreviated by that drug. When bupivacaine is applied at the incision site, both the immediate increase and the persistent aspect are attenuated, the first effect likely due to local anesthetic blockade of afferent impulses normally arising at the injury site. These observations suggest two separable phases of postincisional pain; systemic bupivacaine does not alter the induction phase but selectively inhibits the maintenance phase. Because local bupivacaine reduces both phases, we hypothesize that the induction phase represents a sensitization of peripheral nerve from injury and inflammation at the wound, whereas the maintenance phase represents central sensitization of cells in the spinal cord.[33]

Comparison with Other Models

Our findings share many of the features reported by others in models of chemical[15,34] or thermal[35] injury or inflammation[36] and after incision of the glabrous skin of the rat hind paw.[5] The last of these, a postoperative pain model introduced by Brennan *et al.*,[5] used an incision through skin and fascia of the plantar aspect of the rat's hind paw, sometimes including incision of the more proximal gastrocnemius muscle to induce secondary hyperalgesia, with the nocifensive withdrawal response of the paw to mechanical stimulation by VFH used to determine the pain threshold. Mechanical threshold at the site of the plantar incision was reduced for at least 3 days, with smaller and briefer changes measured approximately 1 cm from the incision. These changes in primary and secondary hyperalgesia correspond to altered weight-bearing of the injured paw in these rats and also resemble certain movement-induced or tactile-evoked pain experienced by patients after surgery.[17,37]

An experimental incision through dermis, fascia, and underlying muscle in human hairy skin produces similar tactile hyperalgesia, accompanied by spreading flare and sensitization of more distant skin that are characteristic of neurogenic and secondary hyperalgesia.[37,38] These are the same general characteristics of surgically induced pain in humans,[38,39] implying that the trauma of the incision is by itself capable of producing the full spectrum of cutaneous hyperalgesic responses.

The rat paw incision model of Brennan *et al.*[5] differs from the one described here in certain quantitative measures. The CTMR remains increased for at least 1 week on the rat's back, minimally twice as long as the signs of

postincisional hyperalgesia in the paw. Secondary allodynia and hyperalgesia from skin incision are also substantially greater on the back, and the threshold forces for response, both before and after surgery, are much lower for the response of hairy skin. These differences could be due to the differences in the length or depth of the incision or the anatomy and innervation of the skin between the two locations; not only is the hairy skin of the back thinner than that of the paw, but it also has a different vascular response to injury due to the related changes in vasoactive regulatory peptides, some of which are also algogenic agents.[8] Alternatively, the longer duration of allodynia on the hairy back after incision could be a result of differences between test procedures. Skin on the back was routinely prodded by VFHs that were well above the threshold for CTMR and considered irritating to several of the investigators when applied to their volar forearm, whereas VFHs applied to the paw only just exceeded withdrawal threshold and were possibly less exacerbating of the postincisional tenderness and thus less likely to extend the period of sensitization.

Which Sensory Neurons Are Involved in Postincisional Pain Changes?

Electrophysiologic experiments indicate that the CTMR occurs in response to activation of both cutaneous Aδ and C fibers,[40] but which of these or other fiber types contributes to postincisional allodynia and, perhaps differently, to hyperalgesia is not known. Physiologically stimulated impulse activity in approximately one fourth of recorded mechanosensitive Aδ- and C-fiber afferents innervating the rat's paw was increased 45 min and 1 day after paw incision, although the firing of low-threshold Aβ-mechanoreceptive fibers at 45 min was unaffected.[29,30] Unfortunately, response properties of small- or large-diameter afferents at longer times after incision have not been reported. Furthermore, the cellular activities that cause the early induction of hyperesthesia after incision may differ from the ones that support the later, sustained effects, and a complete understanding of the overall phenomenon should examine events that contribute at both of these times.

Actions of Bupivacaine

Bupivacaine injected under the skin immediately beneath the locus of the incision or at a distant site was able to suppress primary allodynia and to abolish secondary allodynia, with insignificant effects on hyperalgesia measured by strong VFHs. This result would confirm our initial hypothesis that a brief blockade of afferent impulses arising from the injured skin prevents the long-term development of hyperalgesia, but only if it is known that subcutaneous bupivacaine suppresses the injury-related firing at the incision. However, it is also possible that the antiallodynic effect of bupivacaine occurs through its systemic rather than its local actions, which would preclude a test of the initial hypothesis.

In the paw incision model, a bupivacaine injection (0.5%) given in the skin (0.3 ml) and around the distal nerve (0.2 ml) innervating the incision site 15 min before surgery reduced the change in withdrawal threshold for the first 4 h after incision when compared with vehicle, but this effect had dissipated after 1 day.[31] Intrathecal delivery of 20 μl bupivacaine, 1%, before or after footpad incision, did not modify the hyperalgesic response.[41] These effects are far briefer than the persistent antiallodynic effect of subcutaneous bupivacaine in the current study, despite the lower dose of bupivacaine given here (1.5 *vs.* 2.5 mg for the paw), suggesting that local resorption of the drug may differ between glabrous and hairy skin or that the mechanisms of allodynia and hyperalgesia differ in some bupivacaine-sensitive manner and, specifically, that the spinal cord is not the sole locus of the antiallodynic actions of bupivacaine.

Analogous results were found for the experimental incision model of hairy skin on the human volar forearm. Subcutaneous lidocaine (1%) given at the incision site before surgery reduced the spontaneous pain that immediately followed the incision and suppressed flare formation and secondary hyperalgesia.[38] Primary hyperalgesia was also reduced, but only for a short time after the incision. Lidocaine given at this site 30 min after surgery had none of these effects.[38] Preoperative systemic lidocaine, in contrast, had no effect on the pain of incision while suppressing the formation of the flare and, notably, inhibiting secondary hyperalgesia for several hours, well after the intravenous drug infusion had stopped.[39] Postoperative infusions with intravenous lidocaine produced only transient relief of primary and secondary hyperalgesia, which ended when drug infusion stopped.

Insult and injury to peripheral tissues, *i.e.*, trauma damage and inflammation, can sensitize the peripheral nerves and the CNS through local responses to released inflammatory mediators and the acute activation of nociceptive pathways, as exemplified by the enhanced responses of spinal dorsal horn neurons to moderate strength stimuli after a robust, prolonged activation of C nociceptors.[32,42] Inhibition of the initial sensitizing process, by blockade of nociceptive transduction, impulse conduction, or neurotransmission in the spinal cord should, in principle, preempt the cascade of events that drive the dorsal horn neuronal plasticity underlying spinal sensitization. Published studies show that incision of the rat's footpad sensitizes dorsal horn neurons to mechanical stimulation for at least 1 h, for both high-threshold mechanoreceptive dorsal horn cells and the low-threshold wide-dynamic-range neurons that receive inputs from both nociceptors and innocuous afferents.[42,43] However, only the wide-dynamic-range responses could be elicited by VFHs at the postincisional threshold for nocifensive withdrawal, implying that this neuronal activity corresponds to

allodynic behavior. Because our results show a selective suppression of postincisional (particularly secondary) allodynia by bupivacaine, we hypothesize that it acts on the network that sensitizes wide-dynamic-range neurons in the spinal cord.

Is Bupivacaine Acting "Preemptively"?

Although in these experiments bupivacaine was injected 30 min before the incision, its local action as well as its systemic plasma half-life extends for several hours. Therefore, it is not possible to know when the drug is acting and on which postincisional processes, the initial injury discharge from sectioned nerve, the activation/sensitization of injured and neighboring uninjured fibers by locally released mediators, or the processes causing sensitization in the sensory cell soma or in the CNS. These results do not demonstrate an effect that can be rightly called *preemptive*.[44]

Preemptive use of local anesthetics to reduce postincisional hyperalgesia has produced divergent clinical results. Infiltration at the site of incision with local anesthetics before surgery reduces the postsurgical pain responses for as long as 1-2 postoperative days [45–48] but is no more effective than when the same dose is given after the incision.[49] Continuous wound infiltration with local anesthetic is emerging as an effective treatment for postoperative pain,[50,51] although whether such infiltration should begin before or during surgery or only at wound closing has not been investigated.[41,49,52] In general, however, clinical models have mostly failed to reproduce the positive results of preemptive analgesia obtained in animal experiments.[49] Whether this is due to dosing differences, species-dependent drug susceptibilities, or a fundamental difference in the mechanisms that underlie injury-induced hyperalgesia has not been determined. We believe that the model introduced here has many of the features documented in human postoperative pain and that the pharmacologic results obtained with this model will be applicable to treatment of the human condition, including tests to determine the value of local anesthesia during different perioperative phases.

In conclusion, incision of hairy skin of the rat rapidly leads to long-lasting mechanical allodynia and hyperalgesia, both at the incision site (primary) and more distantly (secondary). Allodynia, particularly in the secondary areas, is selectively suppressed for at least a week by preincisional bupivacaine given subcutaneously at the incision site and elsewhere, revealing the effectiveness of this local anesthetic for suppressing postoperative pain through a mechanism other than a direct blockade of afferent discharges at the incision site.

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