Strain and Sex Influence on Pain Sensitivity after Plantar Incision in the Mouse

Ratan K. Banik, M.B.B.S., Ph.D.,* Young Cheol Woo, M.D., Ph.D.,† Soo Seog Park, M.D., Ph.D.,† Timothy J. Brennan, M.D., Ph.D.‡

Background: A small surgical incision in mouse glabrous hind-paw skin induces short-lasting guarding behavior and mechanical and heat hyperalgesia–like behaviors, which imitate human postoperative pain. The increasing popularity of this animal model in drug discovery necessitates the understanding of genetic and sex influence on this animal model.

Methods: The authors examined pain behaviors on DBA2, C57Bl/6, and 129X1/SvJ mice and male and female DBA2 mice before and after plantar incision.

Results: The baseline nociceptive responses of these strains were similar, with a few exceptions. Heat responses were different between DBA2 and C57Bl/6 mice, and responses to one filament, 14.0 mN, were intermittently different. Sex did not greatly influence baseline responses. After plantar incision, these three strains of mice were not different in the development of guarding behaviors. Heat responses were only different on postincision day 3 (129X1/SvJ vs. C57Bl/6 mice); otherwise, they were the same. The responses to the series of von Frey filaments were the same after incision in the three strains. Sex did not influence incision-induced pain behaviors in DBA2 mice.

Conclusion: Although several studies postulated that mouse strain influences pain models, the authors’ data indicate that such influence on incisional pain is negligible. This suggests that studies using an incision and knockout mice resulting from 129 strain mutation in a C57Bl/6 strain background should have modest influence. The lack of sex differences in incisional pain may encourage researchers to use both male and female subjects in their studies.

THE interindividual variations in pain sensitivity in humans are well known, and postoperative pain is no exception. There is a large variability in postoperative pain intensity after thoracotomy, with 45–65% of patients reporting severe pain and 25–35% of patients reporting mild to moderate pain. Pain research has greatly relied on animal models to understand mechanisms and advance new therapies. However, it is often difficult to reach firm conclusions because of the differences between rodent and humans. Furthermore, even within species, pain-related behaviors are dependent on variables such as strain, sex, and the nociceptive test used. It has recently been recognized that laboratory animals used in pain research have strong genetic predispositions that affect the variability of responses to disease and treatments. The mouse is increasingly popular as a subject in pain studies because gene knockdown technology is available in this species. In addition, mouse strains can be used to identify genes associated with phenotype variability. Mogil et al. recently showed that mice display large differences in behavioral pain sensitivity using a variety of pain assays, injuries, and drug treatments.

During the development of knockout mice, embryonic stem cells of 129 mouse strains are injected into the blastocyst of C57Bl/6 mouse strains. Further generations of congenic knockout mice are derived from multiple breeding with the C57Bl/6 mouse. Lariviere et al. reported that the 129 and C57Bl/6 strains are at opposite ends of the spectrum of pain assays. This creates a concern among pain researchers for the use of knockout mice, which are in fact a 129 strain mutation on a C57Bl/6 genetic background. If these two mouse strains are phenotypically different, differences between knockout versus control mice could be due to the deleted gene interest and the differences between the background 129 and C57Bl/6 strains. Therefore, in this study, we compared baseline pain sensitivity and pain behaviors caused by plantar incision in 129X1/SvJ and C57Bl/6 mice. Because the DBA/2 strain has been shown to have a moderate phenotype among 12 mouse strains and has been suggested to be “representative” of mice, we also studied this strain.

Many studies have identified a sex influence on pain, especially in responses to analgesic drugs. Sex differences in the clinical postoperative pain experience are also evident. To further evaluate sex and postoperative pain, we compared incisional pain behaviors in male and female DBA2 mice.

Materials and Methods

The experimental protocols were approved by the Institutional Animal Care and Use Committee, Iowa City, Iowa.

Mice

A total of 96 mice were used in this study. Sixteen male mice from each strain of DBA2, C57Bl/6, and 129X1/SvJ were used to determine strain differences in the guarding behavior, paw withdrawal latency to radiant heat, and paw withdrawal frequencies to von Frey hair stimulation. To determine sex difference in the DBA2 strain, 24 male and 24 female mice were tested for three behaviors. Among 11 substrains of 129 mice, 129X1/SvJ was chosen because it has been used in a large number

* Assistant Research Scientist, † Visiting Professor, ‡ Professor.

Received from the Departments of Anesthesia and Pharmacology, University of Iowa Hospitals and Clinics, University of Iowa, Iowa City, Iowa. Submitted for publication February 8, 2006. Accepted for publication July 27, 2006. Supported by the Department of Anesthesia at the University of Iowa and grant Nos. GM-55831 and GM-067752 (to T.J.B.) from the National Institutes of Health, Bethesda, Maryland. Srinivasa N. Raja, M.D., served as Handling Editor for this article.

Address correspondence to Dr. Banik: Department of Anesthesia, 3000 ML, University of Iowa Hospitals and Clinics, Iowa City, Iowa 52242. ratan-banik@uiowa.edu Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.
of experiments and is the strain of choice for some embryonic cell lines. This strain was renamed from 129/SvJ in November 2000, because of historical genetic contamination.

§ C57Bl/6 and 129X1/SvJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and DBA2 mice were purchased from Harlan World Headquarters (Indianapolis, IN).

Plantar Incision

Anesthesia was induced by placing animals in a plastic box that contained 4% halothane in air. After loss of righting reflex, anesthesia was maintained by administering 2–3% halothane in air through a tightly fitting mask. An 8-mm longitudinal incision was made with a number 11 blade through the skin, fascia, and muscle of the right hind paw, modified from Pogatzki and Raja7 (fig. 1). The skin was apposed with two single sutures of 6-0 nylon, and the wound site was covered with triple antibiotic ointment. After surgery, anesthesia was discontinued, and the animals were allowed to recover in their cages. At the second postoperative day, sutures were removed during brief anesthesia. Pain behaviors were measured before incision and 4 h, 1 day, 2 days, 3 days, 5 days, and 7 days after incision.

Guarding Behaviors

A cumulative pain score was used to assess nonevoked pain behaviors as described previously for rats.8 Unrestrained mice were placed on a stainless steel mesh floor (4 × 4 mm). Using an angled magnifying mirror, the incised and nonincised paws were viewed. Both paws of each animal were closely observed during a 1-min period repeated every 5 min for 1 h. Depending on the position in which each paw was found during the majority of the 1-min scoring period, a score of 0, 1, or 2 was given. Full weight bearing of the paw (score = 0) was present if the wound was blanched or distorted by the mesh. If the paw was completely off the mesh, a score of 2 was recorded. If the area of the wound touched the mesh without blanching or distorting, a 1 was given. The sum of the 12 scores (0-24) obtained during the 1-h session for each paw was obtained. The difference between the scores from the incised paw and nonincised paw was the cumulative pain score for that 1-h period.

Responses to Mechanical Stimuli

After 1 to 2 h of recovery in clean bedding, the mouse was placed on an elevated stainless steel floor covered with a clear plastic cage top. The animals were allowed to ambulate, explore, and eventually rest lying on the mesh. For testing pain behaviors, von Frey filaments (thin plastic filaments with calibrated forces) were applied adjacent to the wound (fig. 1) from least to greatest forces (0.7-, 2.0-, 3.1-, 5.9-, 14.0-, and 26.4-mN forces). Each filament was applied five times, with 30 s between applications. Data are expressed as the percent of paw withdrawal for each filament. Von Frey filaments have been used to assess mechanical sensitivity of the surgical incision in patients.

Responses to Heat

Withdrawal latencies to heat were assessed by applying a focused radiant heat source in unrestrained mice placed on a heat-tempered glass floor. The heat stimulus is a light from a 50-W projector lamp, with an aperture diameter of 6 mm, applied from underneath a heat-tempered glass floor (3 mm thick) on the middle of the incision (fig. 1). Paw withdrawal latencies were measured to the nearest 0.1 s. The latency time to evoke a withdrawal was determined with a cutoff value of 30 s. The intensity of the heat was adjusted to produce withdrawal latency in normal mice of 25–30 s. Three trials 5–10 min apart were used to obtain average paw withdrawal latency.

Experimental Protocol

Eight mice were tested at one time. Four mice from two of the C57Bl/6, DBA2, and 129X1/SvJ strains were tested
together. In separate experiments, four male and female DBA2 mice were tested together. In our experiments, three nociceptive tests could not be completed between our testing period, 8:00 AM to 1:00 PM. Therefore, no more than two nociceptive tests were performed on each mouse. Testing of guarding pain behavior was always first followed by testing of heat responses. In other groups of animals, only mechanosensitivity was tested.

One person performed all of the experiments. This person was blinded to the strain or sex of mice. However, blinding was limited among strains because of differences in mouse color. All mice were acclimated for 2 days before recording pain measurements. On acclimation days, they were placed in the testing area and allowed to habituate to the new environment. No testing was done on these 2 days. Initially, mice displayed frequent grooming and exploratory behaviors, which were comparatively less in the following days. In the third, fourth, and fifth days of the same week, mice were tested for pain behaviors, and these were regarded as baseline responses.

On the following week, an incision was made in all mice, and 4 h after incision, pain behaviors were tested. The pain behaviors were also tested on the first, second, third, fifth, and seventh postincision days.

Statistical Analysis
Data are expressed as median ± interquartile range for guarding behavior score and mean ± SD for heat latency and response frequency to each von Frey filament. All behavioral data were analyzed both before and after incision using a repeated-measures two-way analysis of variance. If there was a group effect or an interaction between groups and time, a post hoc t test with Bonferroni correction was performed to determine differences among strains. A P value less than 0.05 was considered significant. A statistical result is reported in the text when significant differences (P < 0.05) in both two-way analysis of variance and post hoc t test with Bonferroni correction occurred.

Results
Baseline Pain Sensitivity
The mice did not show any preincision guarding behaviors (figs. 2A). There were differences in heat responses among the three strains (F2,23 = 18.29, P < 0.0001). Post hoc analysis showed that DBA2 mice were significantly different from the C57Bl/6 mice on each day (P < 0.05; fig. 2B); all other comparisons among groups were not different. Analysis of the guarding behaviors (fig. 2C) and heat responses (fig. 2D) in male and female DBA2 mice showed no differences.

The mice from all three strains were insensitive to weak von Frey filament application (0.7 and 2.0 mN). The response frequency was always approximately 0% (data not shown). Similarly, the intermediate filaments

Fig. 2. Influences of strain and sex on baseline (before incision) guarding behavior and heat withdrawal for 3 consecutive days. (A) Baseline guarding in three strains of mice. (B) Baseline heat withdrawal in three strains (F2,23 = 18.29, P < 0.0001). * P < 0.05, Bonferroni post hoc test, versus DBA2. (C) Baseline guarding in male and female DBA2 mice. (D) Baseline heat withdrawal in male and female DBA2 mice. The guarding scores are expressed as the median, and the vertical lines are the 25th and 75th quartiles. The heat withdrawal responses are expressed as the mean, and the vertical lines are SDs.
(3.1 and 6.0 mN) produced response frequencies of less than 10%, and their responses were not different among strains (data not shown). Stronger filaments (14.0 and 26.4 mN) elicited withdrawal frequencies less than 50% in all three strains (figs. 3A and B). A significant difference among groups was present (F2,23 = 6.86, P < 0.005, Bonferroni post hoc test, versus DBA2. † P < 0.05, Bonferroni post hoc test, versus 129X1/SvJ). (B) Response frequency to the 26.4-mN filament in three strains. (C) Response frequency to the 14.0-mN filament in male and female DBA2 mice. (D) Response frequency to the 26.4-mN filament in male and female DBA2 mice.

Fig. 3. Influences of strain and sex on baseline mechanosensitivity for 3 consecutive days. (A) Response frequency to the 14.0-mN filament in three strains of mice (F2,23 = 6.86, P < 0.005). * P < 0.05, Bonferroni post hoc test, versus DBA2. † P < 0.05, Bonferroni post hoc test, versus 129X1/SvJ. (B) Response frequency to the 26.4-mN filament in three strains. (C) Response frequency to the 14.0-mN filament in male and female DBA2 mice. (D) Response frequency to the 26.4-mN filament in male and female DBA2 mice.

(3.1 and 6.0 mN) produced response frequencies of less than 10%, and their responses were not different among strains (data not shown). Stronger filaments (14.0 and 26.4 mN) elicited withdrawal frequencies less than 50% in all three strains (figs. 3A and B). A significant difference among groups was present (F2,23 = 6.86, P < 0.005) in the responses to 14.0-mN von Frey hair stimulation. Post hoc analysis showed that the 129X1/SvJ mice were less responsive than the other two strains (P < 0.05). Analysis of the responses to the 26.4-mN filament in the three strains showed no significant differences among strains.

No difference was observed in the mechanical responses (figs. 3C and D) in male and female DBA2 mice to the 14.0- or 26.4-mN von Frey filament.

Postincision Strain and Sex Influence

The median guarding scores in DBA2, C57Bl/6, and 129X1/SvJ mice were not statistically different for any single day (fig. 4A). The paw withdrawal latencies of DBA2, C57Bl/6, and 129X1/SvJ mice decreased from 25–28 s at baseline (fig. 2B) to 3–4 s at 4 h after incision, to 4–5 s at 1 day after incision and to 6–8 s at 2 days after incision (fig. 4B). By 7 days after incision, paw withdrawal latencies returned to the baseline values. A significant group effect was present (F2,23 = 5.82, P < 0.05); only the latencies on the third day after incision, however, were significant (P < 0.05, C57Bl/6 vs. 129X1/ SvJ). After incision, the median guarding scores were the same in male and female DBA2 mice (fig. 4C). The mean paw withdrawal latencies in male and female DBA2 mice were not significantly different (fig. 4D).

Four hours after incision, DBA2, C57Bl/6, and 129X1/ SvJ mice showed 35–40% responsiveness to the 0.7-mN von Frey filament, respectively, and 45–65% responsiveness to the 1.9-mN von Frey filament (figs. 3A–F). There were unresponsive to these filaments before incision. This suggests that the responsiveness to these filaments could be interpreted as mechanoallodynia-like behavior. However, this was similar in all three mouse strains. The responses to intermediate filaments to mild and stronger filaments, namely 3.1 and 6.0 mN, were similar among the three strains. The sensitivity to stronger von Frey filaments (14.0 and 26.4 mN) changed from less than 50% before incision responses to 100% responsiveness 4 h after incision. These hyperalgesic-like responses were also similar in all three mouse strains. The paw withdrawal frequencies to von Frey filament stimulation (figs. 6A–F) also were not different between sexes.

Discussion

An incision in mice plantar skin produces similar pain behaviors in DBA2, C57Bl/6, and 129X1/SvJ mice. There were few guarding behaviors compared with those reported in rats. This is an important limitation of the mouse model of incisional pain. Our observations indi-
cate that the influence of strain and sex on this mouse model of persistent pain is not as great as that reported in other models and other nociceptive tests.

The Mouse Model of Incision-induced Pain

Pogatzki and Raja\(^7\) previously described the mouse model of incisional pain, which was adapted from the rat model\(^9\) characterized in our laboratory. In the current study, we modified this model by using a longer incision (0.8 mm instead of 0.5 mm) and two sutures. Importantly, we attempted to detect guarding behavior in this model, which was not previously measured. Like others,\(^10,11,12\) we have argued that guarding behavior is spontaneous, non-evoked pain behavior because animals lift their paw without provocation and this behavior was reduced by morphine. This behavior could be related to heat hyperalgesia\(^13\); however, because the animals do not bear weight on the affected paw, mechanical touch might be a factor. In our study, the median guarding behavior score in all three strains of mice was only 6–8, whereas in rats, these scores were 15–24.\(^4,5,10,11\) (B) Heat withdrawal latency in three strains (F\(_{2,25} = 5.82, P < 0.05\)), \(\dagger P < 0.05\), Bonferroni post hoc test, versus 129X1/SvJ, on postoperative day 3 only. (C) Guarding pain score in male and female DBA2 mice. (D) Heat withdrawal latency in male and female DBA2 mice.

Strain Influence on Normal Pain Sensitivity

An important question we asked in this study is, are the C57Bl/6 and 129 strains, which are background strains for producing knockout mice, different in basal pain sensitivity before incision? As discussed in the introduction, current transgenic knockout mice are in fact hybrids of C57Bl/6 and 129 strains. The current data show that these two strains differ somewhat in basal mechanosensitivity but not in heat sensitivity. This finding is partly conflicting with data reported by Lariviere et al.\(^4\) that showed that 129 and C57Bl/6 are highly dissimilar to each other in 13 nociceptive tests, including 4 different heat sensitivity tests. However, our data are in agreement with Varnado-Rhodes et al.,\(^17\) who found that the nociceptive responses for C57Bl/6 and 129 mice in the hind-paw withdrawal test used in the current study and hot-plate test were not different; however, they did differ in their tail-flick responses. The significant difference in the tail-flick responses was eliminated in mice adapted to the testing conditions. The need to perform repeated tests of animal behavior is also illustrated in Miczek et al.\(^18\) These authors found that levels of aggression in Swiss-Webster mice were variable in initial confrontation, but it declined and was less variable after repeated experience.

Lariviere et al.\(^4\) also proposed that DBA2 mice are an intermediate phenotype between the C57Bl/6 and 129 strains. Our data show that for heat sensitivity, DBA2 was slightly different from the C57Bl/6, and for mechanosensitivity, the 129X1/SvJ mice had intermittent tests showing lesser mechanosensitivity.
Sex Influence on Normal Pain Sensitivity

Sex influence on pain sensitivity is of great interest to pain research. An important factor, however, could be the animal strain studied for sex differences, because it is possible that sex differences might be present in some strains but not in others. In the current study, male and female DBA2 mice showed similar pain behaviors before incision. The data from female DBA2 mice were collected without tracking the estrous cycle. Although some studies proposed that the estrous cycle must be taken into account when studying sex differences, in a recent review, Mogil et al. showed that compared with males, there is no evidence of greater variability in pain sensitivity of female mice when the estrous cycle was not determined. Our data are consistent with those of Mogil et al., which showed no sex differences in hot-plate latencies of DBA2 or C57Bl/6 mice. It should be noted, however, that sex differences reported in most of the studies were small. For example, the difference in baseline latencies between sexes on the 49°C tail withdrawal test was reported to be “highly significant,” although the average latencies were 3.2 s in males and 2.9 s in females.

Strain Dependence of Incisional Pain Hypersensitivity

The incision similarly affected pain behaviors of all three mouse strains we studied. This seemed to be unusual because some studies reported differences in other pain models using these strains. However, there is a wide variation in the literature. For example, Mogil et al. first reported that C57Bl/6 and 129 were different in the duration of paw licking in both initial (0–10 min) and late phases (10–60 min) of formalin-induced inflammation, but later the authors reported that these two strains were different only in the late phases, not in the first phases.

Fig. 5. Time course of incisional mechanical allodynia and hyperalgesia in three strains of mice. Response frequencies to the 0.7-mN (A), 1.9-mN (B), 3.1-mN (C), 6.0-mN (D), 14.0-mN (E), and 26.4-mN (F) filaments are similar between strains.
However, although 129 and C57Bl/6 mice showed large differences in the above studies in formalin- and bee venom–induced inflammation,4 they showed no differences in the hypolocomotion after cyclophosphamide-induced cystitis.25 Moreover, these two strains have also been shown to be equally responsive in the development of paclitaxel-induced neuropathy27 and spinal nerve ligation–induced neuropathy.26

The lack of strain difference after incision may be somewhat unique to this model. It may be maladaptive to respond poorly to an injury like an incision; therefore, to respond with significant protective responses after incisional injury may be important for sustaining the strain, i.e., a decreased response may be a negative selection factor for strain viability. Studies hypothesized that an incision has components of both inflammation and neuropathic pain.28 For example, nonsteroidal antiinflammatory drugs have been shown to be effective in animal models of inflammatory pain, whereas gabapentin was reportedly effective in animal models of neuropathic pain. The partial efficacy of both nonsteroidal antiinflammatory drugs and gabapentin was observed in the incisional pain model, suggesting that both inflammatory and neuropathic mechanisms may contribute to the hyperalgesia and allodynia of this model.28 This is supported by Pogatzki et al.,29 who showed capsaicin receptor TrpV1 expression was increased in Freund complete adjuvant–induced inflammation but not in the incision model. The response to antiinflammatory agents,30 spinal N-methyl-D-aspartate receptor blockade,31 spinal non-N-methyl-D-aspartate receptor blockade,15 and parenteral P2X1/3 receptor antagonists32 are different in incisional pain compared with other models.
Sex Influence on Incisional Pain Hypersensitivity

Sex plays a minimal role in pain behaviors after incisional injury in DBA2 mice. This is the first study that evaluated the influence of sex on incisional pain in the mouse. Mechanical sensitivity after incision in male and female Sprague-Dawley rats is also similar. There are sex differences in postoperative pain from human studies. In a recent study, Aubrun et al.6 showed that during intravenous titration of morphine in the postoperative period, women had significantly higher (11% more) visual analog scores and required more morphine than men. Women have greater postoperative pain than men after arthroscopic anterior cruciate ligament reconstruction.35 In contrast, there were reportedly no sex differences in the visual analog scores after either intracranial surgery35 or tooth extraction.36 However, almost all human studies required analgesic use; therefore, comparisons with animal studies are limited. In addition, significant differences are reported in the analgesic drug responses between sexes.37 Perhaps a major difference is the responsiveness of postoperative pain to intraoperative and postoperative opioids.

Significance

The results of these studies demonstrate negligible influence of each of these three strains on incision-induced pain hypersensitivity. This suggests that studies using an incision and knockout mice resulting from 129 strain mutation in a C57BL/6 strain background should have modest influence on pain behaviors. We believe this information will provide important new insights in the perspective of a recent debate on the use of knockout mice in preclinical pain research.3 The lack of sex differences in the current study may encourage pain researchers, who overwhelmingly use male subjects, to include female subjects in their studies without determining the estrous cycle.3

The authors thank Alberto Subieta, B.S. (Research Assistant, Department of Anesthesiology, The University of Iowa, Iowa City, Iowa), for performing the majority of the behavioral experiments.

References