ANESTHESIOLOGY

Morphine Exacerbates Postfracture Nociceptive Sensitization, Functional Impairment, and Microglial Activation in Mice

Wen-Wu Li, Ph.D., Karen-Amanda Irvine, Ph.D., Peyman Sahbaie, M.D., Tian-Zhi Guo, M.D., Xiao-you Shi, M.D., Vivianne L. Tawfik, M.D., Ph.D., Wade S. Kingery, M.D., J. David Clark, M.D., Ph.D.

Anesthesiology 2019; 130:292–308

ABSTRACT

Background: Emerging evidence suggests that opioid use immediately after surgery and trauma may worsen outcomes. In these studies, the authors aimed to determine whether morphine administered for a clinically relevant time period (7 days) in a tibia fracture orthopedic surgery model had adverse effects on postoperative recovery.

Methods: Mice were given morphine twice daily for 7 days after unilateral tibial fracture and intramedullary pin fixation to model orthopedic surgery and limb trauma. Mechanical allodynia, limb-specific weight bearing, gait changes, memory, and anxiety were measured after injury. In addition, spinal cord gene expression changes as well as glial activation were measured. Finally, the authors assessed the effects of a selective Toll-like receptor 4 antagonist, TAK-242, on nociceptive and functional changes after injury.

Results: Tibial fracture caused several weeks of mechanical nociceptive sensitization ($F_{(1, 216)} = 573.38$, $P < 0.001$, fracture + vehicle *vs.* sham + vehicle, n = 10 per group), and this change was exacerbated by the perioperative administration of morphine ($F_{(1, 216)} = 71.61$, $P < 0.001$, fracture + morphine *vs.* fracture + vehicle, n = 10 per group). In additional testing, injured limb weight bearing, gait, and object location memory were worse in morphine-treated fracture mice than in untreated fracture mice. Postfracture expression levels of several genes previously associated with opioid-induced hyperalgesia, including brain-derived neurotrophic factor and prodynorphin, were unchanged, but neuroinflammation involving Toll-like receptor 4 receptor–expressing microglia was observed (6.8 ± 1.5 [mean ± SD] cells per high-power field for fracture + vehicle *vs.* 12 ± 2.8 fracture + morphine, $P < 0.001$, n = 8 per /group). Treatment with a Toll-like receptor 4 antagonist TAK242 improved nociceptive sensitization for about 2 weeks in morphine-treated fracture mice ($F_{(1, 198)} = 73.36$, $P < 0.001$, fracture + morphine + TAK242 *vs.* fracture + morphine, n = 10 per group).

Conclusions: Morphine treatment beginning at the time of injury impairs nociceptive recovery and other outcomes. Measures preventing glial activation through Toll-like receptor 4 signaling may reduce the adverse consequences of postoperative opioid administration.

(Anesthesiology 2019; 130:292–308)

EDITOR'S PERSPECTIVE

What We Already Know about This Topic

- Opiates, including morphine, remain a key component in the management of postsurgical pain; however, perioperative use of opiates is associated with a slower resolution of pain and functional status
- It is possible that the delayed recovery attendant with the administration of morphine may be attributable to morphine-induced enhancement of neuroinflammation

What This Article Tells Us That Is New

- In a mouse tibia fracture and intramedullary pinning model, injury-induced allodynia and neuroinflammation, in particular microglial activation, were significantly increased by morphine
- Reduction of microglial activation by an antagonist of the Toll-like receptor 4 attenuated the adverse effects of morphine
- The data are consistent with the premise that morphine increases nociceptive sensitization, functional impairment, and prolongs recovery; suppression of neuroinflammation, and in particular microglial activation, can mitigate the adverse effects of morphine

Opioids remain the mainstay of treatment for severe acute pain attributable to surgery and trauma, despite rising concerns over the consequences of even short-term opioid administration. Apart from acute side effects, epidemiologic studies suggest that the aggressive use of opioids in the acute setting after injury is associated with poorer long-term outcomes and a higher likelihood of chronic opioid use.[1,2] Exposure to opioids at the time of surgery or traumatic injury is a common initiating event leading to long-term dependence and addiction.[3,4] Even better established are the poorer outcomes of surgery among patients chronically consuming opioids before their procedures.[5] For example, lower functional scores, higher complications rates, and the more common need for revision has been shown for opioid consuming patients undergoing total knee arthroplasty.[6] Results from laboratory investigations show that animals given opioids, particularly morphine, near the time of incision, peripheral nerve injury, or spinal cord injury recover normal nociceptive sensitivity more slowly than do opioid-naïve animals.[7–11] Identifying the long-term consequences of opioid administration after surgery and other types of injury is important to defining their proper use. Ultimately, information relating dose and duration of pre- and postoperative opioid exposure to recovery may also be valuable in estimating the utility of multimodal nonopioid approaches to providing analgesia that can be more expensive and complex to administer.

Opioid-induced hyperalgesia, a form of sensitization to noxious stimuli after exposure to opioids, has been described

in laboratory animals, in humans after the short-term administration of potent intravenous opioids, and in pain patients given opioids chronically for control of their symptoms.[12–14] Opioid-induced hyperalgesia may, therefore, help to explain the association between perioperative opioid exposure and poorer outcomes. For example, animals with hind paw incisions given several days of morphine treatment showed slower nociceptive recovery from these minor injuries, and elevations in the expression of several pain-related genes, including brain-derived neurotrophic factor and prodynorphin in spinal tissue.[10] Epigenetic mechanisms such as the acetylation of histone proteins were demonstrated to prolong the effects of these morphine–injury interactions. Likewise, rats with nerve injuries recover much more slowly from their injuries if given several days of morphine treatment, effects that were attributed to the activation of spinal microglia and the production of cytokines such as interleukin 1β.[8] Emerging evidence also points to a role of Toll-like receptor 4, a key innate immunity receptor, as the mediator for activation of microglia.[15] Morphine may bind and activate Toll-like receptor 4 in microglia,[16] or glial activation may ultimately be secondary to opioid actions on peripheral sensory neurons.[17] In fact, the dorsal horn of the spinal cord has been posited to be a center of convergence for many proposed mechanisms supporting opioid-induced hyperalgesia.[12,13]

In these studies, we aimed to determine whether a clinically relevant week-long course of opioid treatment, beginning at the time of tibial fracture and surgical pinning, would impair both the nociceptive and functional recovery of the animals from their injuries. We hypothesized that persistent changes in the spinal expression of pain-related genes might contribute to any such observed effects.

Materials and Methods

Animals

All animal experimental protocols were approved by the Veterans Affairs Palo Alto Health Care System Institutional Animal Care and Use Committee (Palo Alto, CA) and followed the animal subjects guidelines of the International Association for the Study of Pain. Male C57BL/6J mice were obtained from The Jackson Laboratory (Sacramento, CA) at 8–10 weeks of age. Husbandry procedures were as described previously.[18] The timeline for experimental procedures is summarized in figure 1.

This article is featured in "This Month in Anesthesiology," page 5A. This article has a video abstract.

Submitted for publication January 18, 2018. Accepted for publication October 1, 2018. From the Anesthesiology Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California, and Department of Anesthesiology, Stanford University School of Medicine, Stanford, California (W.-W.L., K.-A.I., P.S., X.-y.S., J.D.C.); Physical Medicine and Rehabilitation Service, Veterans Affairs Palo Alto Health Care System, Palo Alto, California (T.-Z.G., W.S.K.); Department of Anesthesiology, Perioperative & Pain Medicine, Stanford University School of Medicine, Stanford, California (V.L.T.).

Surgery

The fracture mice underwent right tibial fracture followed by intramedullary pinning to enhance fracture stabilization and allow for early hind limb movement and weight bearing. The intramedullary pinning technique was based on the procedure described by Sigurdsen *et al.*[19] and used recently by our group for rats.[20] To expose the right tibia, an 8-mm-long incision was made in the muscle and skin overlying the anterior aspect of the right proximal tibia. A small hole was drilled through the tibial cortex approximately 2 mm below the tibial plateau and 1 mm medial to the tibial tuberosity. Using a fine-tooth circular metal saw blade mounted on an electric drill, a small notch was then cut into the tibia cortex just proximal distal to the bony union of the tibia and fibula. The distal tibia was manually fractured, and a sterile 27-gauge stainless steel needle was inserted down the medullary canal across the fracture site until the tip of the needle reached the distal tibia. The proximal needle was cut flush to the tibial periosteum. The divided muscle was sutured using 6-0 silk (Henry Schein Inc., USA) and the skin incision closed with Refex 7 wound clips (CellPoint Scientific Inc., USA). Sham-operated mice received anesthesia and skin incisions without tibial fracture.

Drug Treatment Protocols

Repeated Morphine Administration. To test the hypothesis that repeated morphine administration can exacerbate hyperalgesia in animals after a bone injury, tibial fractures were created in mice followed by 7 days of subcutaneous morphine treatment (Sigma-Aldrich, USA). Treatment began immediately after surgery at a dose of 10 mg/kg (subcutaneously), and then 20 mg/kg (subcutaneously), twice daily, for the remaining 6 days (fig. 1). Additional mice received vehicle injections after fracture or sham surgeries. Nonfracture mice received vehicle or morphine alone. The dosages of morphine were selected based on previous experiments evaluating opioid-induced hyperalgesia.[9,21,22]

Prostaglandin E2 Administration to Detect Latent Nociceptive Sensitization. To evaluate whether 7 days of morphine treatment, tibial fracture, or a combination of the two caused latent nociceptive sensitization, prostaglandin E2 was administered subcutaneously 53 days after fracture or sham surgery when hind paw allodynia had recovered fully. Briefly, prostaglandin E2 or vehicle (0.1% ethanol in 0.9% saline) was injected subcutaneously into the plantar surface of the fracture hind paw in a volume of 15 μl. Nociceptive von Frey testing was performed before and at 1, 4, 24, 48, and 72 h after injection (fig. 1). The dosage and preparation of prostaglandin E2 used in the present study was based on previous reports.[22]

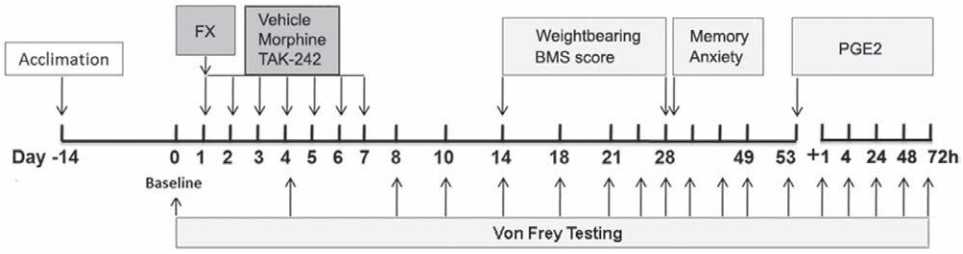

Fig. 1. Timeline for experimental procedures. The schematic illustrates the chronologic order of experiments for tibia fracture and pinning (FX), behavioral tests, drug administration, and functional assessments. TAK-242, Toll-like receptor 4 antagonist; BMS, Basso mouse scale; PGE2, prostaglandin E2.

Toll-like Receptor 4 Blockade Using TAK-242. TAK-242, a systemically available selective inhibitor of Toll-like receptor 4 signaling, was used to determine whether repeated morphine administration amplifies mechanical allodynia *via* Toll-like receptor 4 in the setting of limb trauma. TAK-242 was purchased from MedChem (HY-11109, USA) and freshly prepared in dimethyl sulfoxide (Sigma) as a stock solution (50 mg/ml), which was further diluted in sterile water (0.5 mg/ml with 1% dimethyl sulfoxide) just before use. After fracture with pinning, TAK-242 (3 mg/kg, intraperitoneally) was administered once daily concurrently with the morning morphine dose for 7 days after fracture (fig. 1). Fracture mice treated with morphine or vehicle and sham-operated mice were also given TAK-242 or vehicle as controls. The dose of TAK-242 used in the present study was based on previous reports.[23,24]

Hind Paw Nociceptive Testing

To measure mechanical allodynia in the mice, an up-down von Frey testing paradigm[25] was used (fig. 1), as previously described.[18,26] Briefly, mice were placed on wire mesh platforms in clear cylindrical plastic enclosures. After 15 min of acclimation, von Frey fibers of sequentially increasing stiffness were applied against the hind paw plantar skin. Withdrawal of or licking the hind paw after fiber application was scored as a response. Estimation of the mechanical withdrawal threshold by data-fitting algorithm permitted the use of parametric statistics for analysis.[27]

An incapacitance test meter (IITC Life Science Inc., USA) was used to measure hind paw unweighting, as previously described.[18] Hind paw weight-bearing data were analyzed as a ratio between the right (R) hind paw weight and the sum of right and left (L) hind paw values ($[2R/(R + L)] \times 100\%$).[18]

Basso Mouse Scale Locomotor Testing

The Basso Mouse Scale[28] was used to evaluate the motor function of mice subjected to tibia fracture at 2 and 4 weeks after injury (fig. 1). The Basso Mouse Scale is a 10-point locomotor rating scale (0 to 9). Animals with normal locomotion achieve a score of 9. The scale evaluates parameters including joint movement, stepping ability, coordination, and trunk stability. Mice were scored by two investigators who were unaware of the experimental groups based on hind-limb movements made in an open field enclosure (36-inch in diameter) during a 4-min interval. The more specifically gait-related Basso Mouse Scale subscore was also used.[28] This subscore quantified improvements in the areas of stepping frequency, coordination, paw position, trunk stability, and tail position.

Open Field, Zero Maze, and Novel Object Recognition Assays

Open Field and Object Memory Tests. Open field testing was completed as described previously.[29] Briefly, the open field arena was made from opaque plastic material and measured 40 × 40 × 40 cm. This was used to assess locomotion, object location, and object recognition working memory tests. The open field test lasted 10 min, during which the total locomotor activity (distance traveled) in the arena and the time spent in the center (11% of total area) were determined.

The day after open field testing, object memory experiments were completed. Initially, mice were presented with two identical objects for 10 min as previously described.[29,30] Then, during a 5-min trial one of the objects was moved to a new location, and exploratory behavior (investigation time) was recorded. Subsequently, mice were returned to home cages for a 5-min period and then were returned to the arena with one of the previous identical objects being replaced with a novel one. Recordings were done for exploratory behavior toward objects under 50 lux luminosity.

Anxiety Assessment. An elevated zero maze test for anxiety measurement was performed as described previously.[29] Luminosity inside the open quadrant was measured to be 50 lux, whereas that inside the closed quadrant was measured to be 20 lux. Each testing session started by placing

the mouse in front of one of the closed quadrants of the maze. Total number of entries to the open quadrants was video recorded and later scored for the 5-min test period.

RNA Extraction and Real-time Polymerase Chain Reaction

For molecular analyses, animals were euthanized by carbon dioxide asphyxiation on day 14 after surgery. The harvested spinal cords were immediately snap-frozen on dry ice and stored at −80°C before RNA isolation. Total RNA from lumbar spinal cord was extracted using the GeneAll Hybrid-R kit (GeneAll Biotechnology, Seoul, South Korea), dissolved in RNase-free water, and the purity and concentration were determined spectrophotometrically. Next, cDNA was synthesized from 1 μg RNA using a RT^2 first strand cDNA Synthesis Kit (Qiagen, USA). Real-time polymerase chain reactions were conducted using RT^2 quantitative polymerase chain reaction Primer Assays (Qiagen) and RT^2 SYBR Green ROX mastermixes (Qiagen). The RT^2 quantitative polymerase chain reaction Primer Assays for mouse included brain-derived neurotrophic factor, prodynorphin, Tachykinin 1, Tachykinin receptor 1, inducible nitric oxide synthase, FBJ murine osteosarcoma viral oncogene homolog B, hydroxytryptamine receptor 3a and β-actin. Polymerase chain reaction component mix for each reaction was a 25-μl final volume of 12.5-μl RT^2 SYBR green mastermix (Qiagen), 1 μl of diluted template, 1 μl RT^2 quantitative polymerase chain reaction Primer Assay (Qiagen), and 10.5 μl of RNase-free water. Real-time polymerase chain reaction amplification of brain-derived neurotrophic factor, prodynorphin, Toll-like receptor 4, and β-actin was performed on an ABI 7900HT sequencing detection system. The data from real-time polymerase chain reaction experiments were analyzed by the comparative cycle threshold method. All analyses were performed in triplicate.

Protein Extraction and Enzyme Immunoassay

The lumbar spinal cord was collected from animals euthanized 14 days after surgery, and the tissue was minced into fine pieces in ice-cold phosphate-buffered saline, pH 7.4, containing a cocktail of protease inhibitors (Roche Applied Science, USA) and followed by homogenization using a Polytron device (Brinkmann Instruments, USA). Homogenates were centrifuged at 12,000*g* for 15 min at 4°C, and supernatant fractions were frozen at −80°C until required for enzyme-linked immunosorbent assay performance. An aliquot was subjected to protein assay (Bio-Rad Laboratories Inc, USA) to normalize mediator levels. Brain-derived neurotrophic factor and prodynorphin protein levels were determined using mouse brain-derived neurotrophic factor (Genway Biotech Inc., USA) and prodynorphin (MyBiosource, USA) enzyme-linked immunosorbent assay kits, respectively. Toll-like receptor 4 protein levels were determined using a mouse sandwich enzyme-linked immunosorbent assay kits (LifeSpan BioSciences Inc., USA) per the manufacturer's instructions. Absorbance of standards and samples was determined spectrophotometrically at 450 nm using a microplate reader (Bio-Rad Laboratories Inc.). Results were plotted against the linear portion of the standard curve, and the protein concentration of each sample was expressed as ng/mg or pg/mg protein of sample.

Tissue Processing and Immunofluorescence Confocal Microscopy

To investigate the effect of chronic morphine treatment on spinal microglial activation and Toll-like receptor 4 expression, mice were euthanized and immediately perfused with 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, *via* the ascending aorta at 2 weeks postfracture. Then lumbar spinal cord between L4-6 was removed and postfixed in 4% paraformaldehyde for overnight at 4°C. Tissue processing was then completed as previously described.[31] The following primary antibodies were used: rat anti-mouse CD11b (clone 5C6), 1:500 (Bio-Rad Laboratories Inc.), polyclonal rabbit anti-Iba1, 1:500 (Wako, Japan), and polyclonal rabbit anti–Toll-like receptor 4 (M-300), 1:1,000 (Santa Cruz Biotechnology, USA). After rinsing the slides in phosphate-buffered saline, single- and double-labeling immunofluorescence was performed using the following secondary antibodies: donkey anti-rat immunoglobulin G conjugated with Alex Flour 488 (1:1,000), donkey anti-rabbit immunoglobulin G conjugated with Alex Flour 488 (1:1,000), or donkey anti-rabbit immunoglobulin G conjugated with Alex Flour 547 (1:1,000; Jackson ImmunoResearch Laboratories, USA). After a final rinse in phosphate-buffered saline, slides were then coverslipped using an anti-fade mounting medium (Invitrogen, USA). Images were obtained using confocal microscopy (Zeiss LSM710, Carl Zeiss, Jena, Germany) and stored on digital media. For CD11b signaling, images were quantified by a blinded investigator for fluorescent intensity using ImageJ software (National Institutes of Health, Bethesda, Maryland). A total of four to six sections of the spinal cord between L4 to L6 were selected from each mouse. Then for each section, four to five high-power (×400 magnification) fields of the superficial layer of the dorsal horn were captured to derive a mean score for that spinal cord. The total area quantified in the dorsal horn of each section was 0.18–0.22 mm^2. The individual mean scores were then used to calculate the mean intensity values and SD of the mean for each group (n = 6 per group for Iba-1, n = 8 per group for CD-11b). The immunohistochemistry data quantified in the figures is presented as mean intensity per high-powered field. Control experiments included incubation of slices in primary and secondary antibody-free solutions, which exhibited low-intensity nonspecific staining patterns in preliminary experiments (data not shown). For Toll-like receptor 4, quantitative studies were based on three or more replicates. The

number of Toll-like receptor 4–positive cells were counted per high-power field (×630) in the dorsal spinal cord of eight mice per group. Control experiments included incubation of slices in primary and secondary antibody-free solutions, both of which led to low-intensity nonspecific staining patterns in preliminary experiments (data not shown).

Statistical Analysis

Animals were randomly assigned to treatment groups at the time of initiation of experiments. Data collection was conducted by observers blind to group assignment or drug treatment. There are two animals missing from the data analysis, both from the fracture + vehicle group, which were found dead on postoperative days 1 and 29 because of unknown reasons.

Behavioral data collected over time after subcutaneous injections of morphine were analyzed by a repeated-measures two-way ANOVA on data for each test time point, comparing various treatment groups, where the repeated measure was time post injection. A Bonferroni test was used to determine the source of differences between each treatment. For open field, zero maze, and novel object recognition assays, all recordings were automatically analyzed in real time by TopScan software (Clever System, USA). For object memory tests, the percent time spent investigating each object *versus* other was compared using a two-way ANOVA followed by Holm–Sidak's *post hoc* tests for each experimental group. Data from open field and zero maze experiments were analyzed by a one-way ANOVA followed by Tukey's *post hoc* test for multiple comparisons. Data from the Basso Mouse Scale score, Basso Mouse Scale subscore, gene expression, and immunohistochemistry experiment were analyzed by a one-way ANOVA followed by a Bonferroni test for multiple comparisons. For simple comparisons of two means, two-tailed *t* test was performed. No *a priori* statistical power calculation was conducted, and group sizes were selected based on experience with this model and tests. During peer review, additional experiments were requested and conducted. The data were reanalyzed after the inclusion of these experiments without additional multiple comparisons adjustment for "interim" analysis. All data are presented as the mean ± SD of the mean, and differences were considered significant at a P value less than 0.05 (Prism 6, GraphPad Software, USA).

Results

Postfracture Morphine Treatment Enhances Nociceptive Sensitization

Our lab previously observed that 4 days of ascending doses of morphine treatment induced opioid-induced hyperalgesia.[9–11,21,22] Here, we examined the effects of 7 days of morphine treatment in mice after tibial fracture and intramedullary pin fixation on mechanical allodynia and weight-bearing. Figure 1 summarizes the experimental timeline, the daily dosing schedule of drugs and behavioral testing for hind paw mechanical allodynia and hind limb unweighting. The data in figure 2 demonstrate that 7 days of morphine administration intensified and lengthened nociceptive sensitization after tibial fracture. Morphine treatment in sham-operated animals resulted in transient von Frey mechanical hypersensitivity that peaked at day 8 and resolved by day 14 ($F_{(1, 216)} = 9.55$, sham + morphine *vs.* sham + vehicle, $P < 0.001$; fig. 2A). The vehicle-treated, fracture mice had significantly lower and more prolonged mechanical thresholds (lasting until day 28 after fracture) when compared with the vehicle-treated, sham mice ($F_{(1, 216)} = 573.38$, $P < 0.001$; fig. 2A). We also observed that the morphine-treated fracture mice exhibited even lower mechanical thresholds and had a slower recovery than the vehicle-treated, fracture mice ($F_{(1, 216)} = 71.61$, $P < 0.001$; fig. 2A). Figure 2B demonstrates the effects of chronic morphine on weight-bearing changes at 2 and 4 weeks after fracture. Fracture mice significantly unweighted the injured limbs at 2 and 4 weeks after fracture ($F_{(1, 36)} = 109.57$, $P < 0.001$; fig. 2B), and morphine treatment exacerbated this fracture-induced unweighting ($F_{(1, 36)} = 36.45$, $P < 0.001$; fig. 2B). These studies demonstrate that a short course of morphine treatment impairs the nociceptive and functional recovery of animals after surgically treated fractures.

Under some conditions, previous injury induces a prolonged period of vulnerability to exaggerated sensitization after subsequent injury termed "hyperalgesic priming."[32] Our previous data and the data of others showed that opioid-induced long-term pain vulnerability may increase pain response after tissue injuries such as hind paw inflammation, incision, or nerve injury.[33–35] We therefore investigated whether chronic exposure to opioids could alter fracture-induced hyperalgesic priming. Mice having previously undergone hind limb fracture or morphine treatment alone demonstrated nociceptive sensitization after prostaglandin E2 injection, similar to control mice (fig. 2C). However, mice that received morphine treatment for 7 days after hind limb fracture showed greater hyperalgesic responses at 48 and 72 h after prostaglandin E2 injection ($F_{(1, 108)} = 29.66$, $P < 0.001$; fig. 2C).

Postfracture Morphine Treatment Impairs Gait Recovery after Fracture

The locomotor ability of both morphine-treated, and vehicle-treated, fracture and sham mice was assessed using the Basso Mouse Scale[28]. As shown in figure 3, Basso Mouse Scale scores and Basso Mouse Scale gait-related subscale scores for the morphine-treated and vehicle-treated fracture mice were significantly lower when compared with morphine-treated or vehicle-treated sham mice, 2 weeks after injury ($F_{(3, 35)} = 37$, $P < 0.001$, fig. 3A; $F_{(3, 35)} = 32$, $P < 0.001$, fig. 3B). Furthermore, morphine-treated fracture mice were

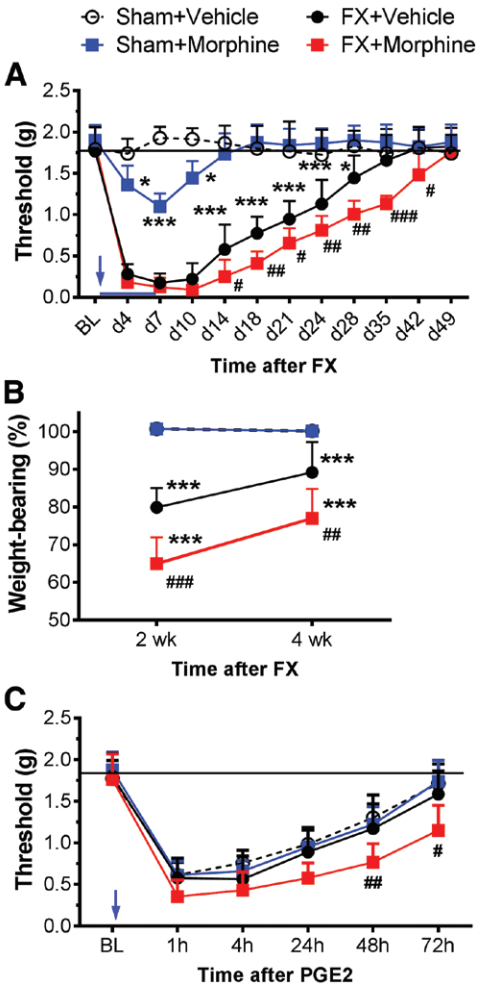

Fig. 2. Repeated morphine administration after fracture/pin (FX) exacerbates nociceptive sensitization and hyperalgesic priming. (*A*) Seven days of morphine treatment in sham-treated control mice induced hind paw von Frey allodynia lasting for 6 days (post FX days 4 to 10). Vehicle treated FX mice developed allodynia lasting 28 days after injury, and 7 days of morphine treatment starting at the time of injury exacerbated and prolonged the hind paw allodynia in FX mice. (*B*) FX reduced hind limb weight bearing at 2 and 4 weeks after injury in vehicle-treated mice, and 7 days of morphine treatment exacerbated the hind limb unweighting in FX mice at both time points. (*C*) Intraplantar injection of prostaglandin E2 (PGE2) at 53 days after injury (after the resolution of allodynia in the vehicle and morphine treated FX mice) caused exacerbated allodynia in the morphine-treated FX mice, relative to vehicle-treated FX mice, morphine-treated sham mice, and vehicle-treated sham mice, indicating enhanced hyperalgesic priming even after the resolution of allodynia in the morphine-treated FX mice. *Arrows* indicate the time points at which morphine or PGE2 were injected. Data are expressed as mean values ± SD, N = 10 per group. #P < 0.05, ##P < 0.01, ###P < 0.001 FX + Vehicle *versus* FX + Morphine. *P < 0.05, **P < 0.01, ***P < 0.001 FX + Vehicle *versus* Sham + Vehicle.

significantly more impaired than the untreated fracture mice with absence of coordination and abnormal rotation of the affected ankle on initial contact or lift off (Basso Mouse Scale score $P < 0.001$, Basso Mouse Scale subscore $P < 0.001$: FX + Morphine *vs.* FX + Vehicle). The Basso Mouse Scale scores and subscores for all the injured mice at 4 weeks after injury were similar to the 2-week time point ($F_{(3, 35)} = 19$, $P < 0.001$, fig. 3C; $F_{(3, 35)} = 19.1$, $P < 0.001$, fig. 3D), regardless of treatment. However, at 4 weeks after injury, there were no longer significant differences in the gate score between morphine-treated and vehicle-treated fracture mice (Basso Mouse Scale score $P = 0.13$, and Basso Mouse Scale subscore $P = 0.07$: FX + Morphine *vs.* FX + Vehicle).

The Effects of Limb Fracture and Morphine Treatment on Memory and Anxiety-related Behaviors

Location memory was intact in both vehicle-treated fracture mice and in morphine-treated sham mice (fig. 4A). However, fracture mice treated after their injuries with morphine had impaired object location memory at 4 weeks after injury ($F_{(3, 118)} = 3.14$, $P < 0.05$). No differences in object recognition memory were seen in any group ($F_{(3, 118)} = 0.86$, $P = 0.46$; fig. 4B).

Assessment of open field locomotor activity in sham or fracture mice with or without morphine treatment revealed no significant differences in the total distance traveled at 4 weeks after fracture ($F_{(3, 59)} = 0.93$, $P = 0.43$; fig. 4C). None of the groups tested displayed differences in time spent in the center of the open field apparatus, evidence that anxiety-related changes were not present at 4 weeks after fracture ($F_{(3, 59)} = 0.35$, $P = 0.79$; fig. 4D). We then investigated anxiety and risk-taking behaviors in the same groups using the complementary elevated zero maze test. No significant differences were observed in the time spent in the open arms ($F_{(3, 55)} = 0.38$, $P = 0.77$) or number of open arm entries ($F_{(3, 55)} = 0.69$, $P = 0.56$) between sham or fracture mice with or without morphine (fig. 4, E and F).

Tibial Fracture and Morphine Treatment Enhance Toll-like Receptor 4 mRNA and Protein Expression in Spinal Cord Tissue, but Do Not Change Spinal Brain-derived Neurotrophic Factor and Prodynorphin Expression

Our laboratory has recently demonstrated that hind paw incision and perioperative opioid administration can cause additive effects on the spinal expression of several pain-related genes, including brain-derived neurotrophic factor and prodynorphin, at least within the first few days after injury.[36–38] In this study, we investigated whether longer term (7 days) administration of morphine in control or tibia fracture mice would show similar expression changes in the spinal cord tissue. It is surprising our results revealed that spinal levels of brain-derived neurotrophic factor (fig. 5, A and B) and prodynorphin (fig. 5, C and D), either at

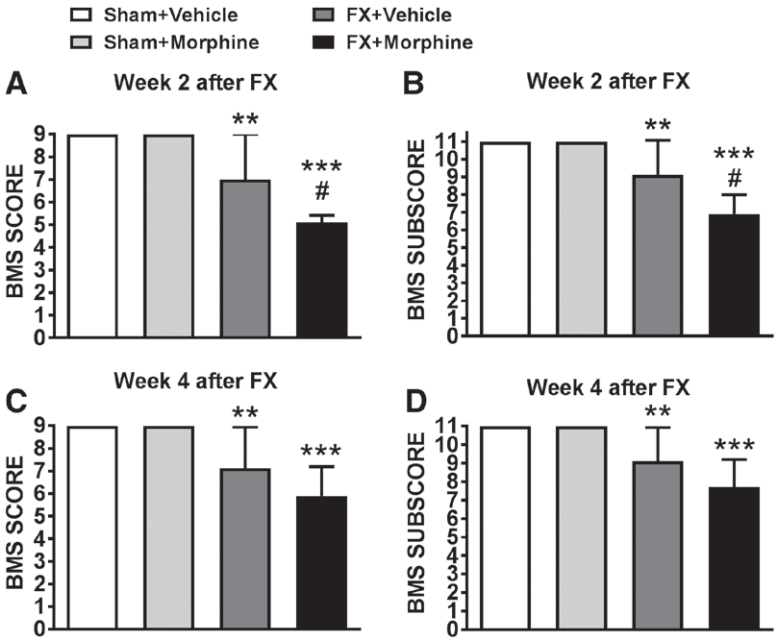

Fig. 3. Morphine treatment exacerbated locomotor impairment after fracture/pin (FX). Locomotor ability was assessed using the Basso mouse scale (BMS) at 2 and 4 weeks after fracture. (*A* and *B*) BMS scores and subscores of both sham and fracture pinning mice treated with and without morphine at 2 weeks after FX. The BMS scores and subscores were significantly lower in the morphine-treated and untreated FX mice compared with morphine-treated and untreated sham mice, respectively. Furthermore, 7-day exposure to morphine in FX mice caused greater locomotor impairment than observed in vehicle-treated FX mice. Morphine treatment had no effect on locomotor function of the sham mice. (*C* and *D*) The BMS scores and subscores for mice at 4 weeks after FX were not significantly different when compared with FX mice at 2 weeks after FX, regardless of treatment. However, at 4 weeks after FX, there was no longer a significant difference in the extent of functional recovery between morphine-treated and vehicle-treated mice. Data are expressed as mean values ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ FX + Vehicle (N = 9) or FX + Morphine (N = 10) *versus* Sham + Vehicle (N = 10), $\#P < 0.05$ FX + Morphine (N = 10) *versus* FX + Vehicle (N = 9).

the mRNA or protein level, were not altered in vehicle-treated or morphine-treated mice with or without tibial fracture despite clear nociceptive and functional changes at 2 weeks post injury (1 week after the completion of morphine treatments). Additional quantitative polymerase chain reaction experiments using the same samples failed to reveal changes in expression of several other pain and opioid adaptation-related genes including Tachykinin 1, Tachykinin receptor 1, inducible nitric oxide synthase, FBJ murine osteosarcoma viral oncogene homolog B, and hydroxytryptamine receptor 3a (data are not shown).

Recent data suggest that Toll-like receptor 4 plays a pivotal role in pain[15,39,40] and opioid-induced hyperalgesia.[7,8,41,42] Therefore, we determined whether spinal Toll-like receptor 4 expression was altered in our experimental paradigm. Using quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assays, we observed that Toll-like receptor 4 is expressed at low levels in the lumbar spinal cord of vehicle-treated, sham surgery mice. Furthermore, morphine treatment of sham-operated mice had no significant effects on Toll-like receptor 4 mRNA and protein expression when measured 1 week after the end of morphine treatments. In contrast, Toll-like receptor 4 mRNA expression was increased in the lumbar spinal cord tissue of fracture mice at 2 weeks after injury, and these increases were significantly enhanced by the administration of morphine ($F_{(3, 36)} = 19$, $P < 0.001$, *post hoc* test: FX + Morphine *vs.* FX + Vehicle, $P < 0.001$; fig. 5E). Increased Toll-like receptor 4 protein expression was also observed as a result of morphine treatment compared with relevant controls ($F_{(3, 36)} = 41$, $P < 0.001$, *post hoc* test: FX + Morphine *vs.* FX + Vehicle, $P < 0.001$; fig. 5F).

Postfracture Morphine Treatment Potentiates Microglia Activation in the Dorsal Horn

It has been suggested that neuroinflammation mediated by Toll-like receptor 4–expressing microglia is a significant contributor to the negative consequences of opioid therapy including opioid-induced hyperalgesia.[43] Our observations of enhanced expression of Toll-like receptor 4 (fig. 5) suggested that spinal microglial neuroinflammation might be present in tibial fracture mice, and that the degree of neuroinflammation could be greater in fracture mice treated with morphine. To measure the extent of microglial

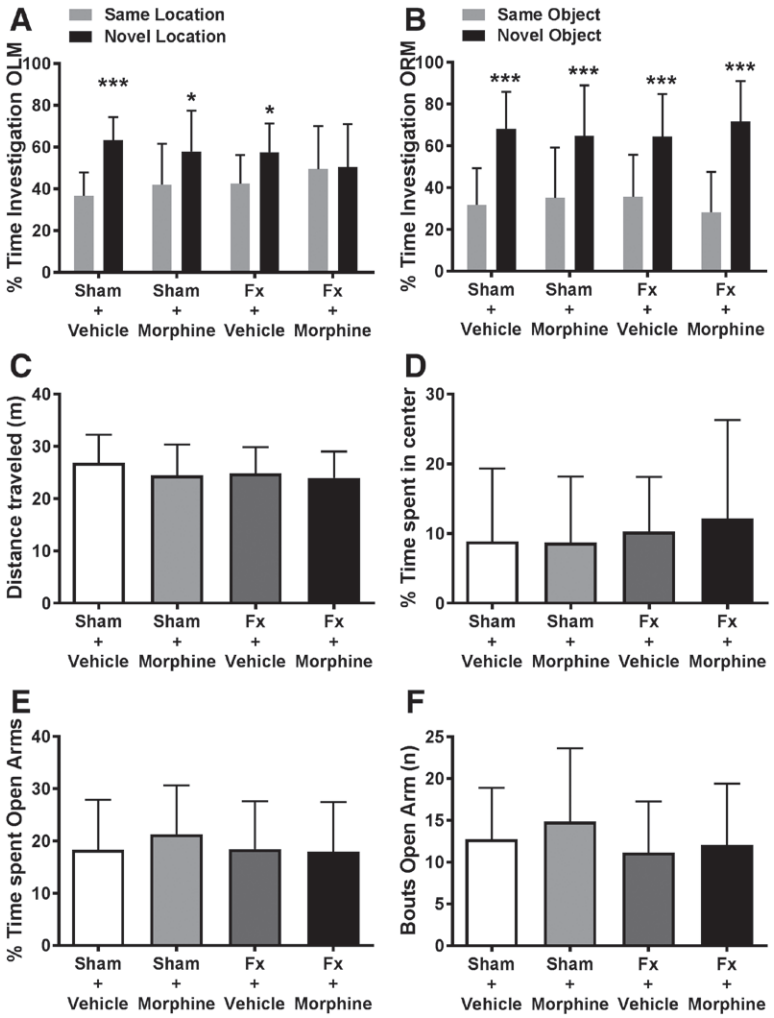

Fig. 4. Evaluation of working memory, open field activity, and anxiety in morphine or vehicle treated fracture/pin (FX) mice at 4 weeks after injury. Object location (OLM) and recognition (ORM) working memory tests were employed, with a 5-min interval between acquisition and retrieval trials. (*A* and *B*) Fracture + morphine-treated mice displayed deficits in spatial OLM, but not in nonspatial ORM, working memory tests. Data were analyzed by two-way ANOVA followed by Holm–Sidak's *post hoc* tests. In the open field test (*C* and *D*), all groups were similar in distance traveled and in percent time spent in the center 11% of the arena. For both tests the following groups were used: Sham + Vehicle (N = 16), Sham + Morphine (N = 16), FX + Vehicle (N = 15), and FX + Morphine (N = 16; *E* and *F*). No differences in the percent time spent or number of entries into the open arms of the zero maze were seen between any of the groups: Sham + Vehicle (N = 15), Sham + Morphine (N = 15), FX + Vehicle (N = 14), and FX + Morphine (N = 15). Data were analyzed by one-way ANOVA followed by Tukey's *post hoc* tests. Data are expressed as mean values ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ for Novel *versus* Same.

activation we used two common markers for both resting and activated microglia, CD11b (integrin alpha M) and IBA-1 (calcium-binding adapter molecule-1). At 1 week after treatment, there were no CD11b and IBA-1 expression differences in vehicle-treated and morphine-treated sham-operated mice (fig. 6). CD11b and IBA-1 microglia were expressed within the dorsal horn and were evenly distributed with a "resting" morphology comprising a small cell body with long, thin processes. At 2 weeks after fracture, IBA-1– and CD11b-labeled microglia in the dorsal horn, ipsilateral to the tibial fracture, had an activated morphology with an enlarged and rounded cell body (*i.e.*, amoeboid shape) with fewer and shorter processes. In contrast, CD11b- and IBA-1–positive microglia within the dorsal horn, contralateral to the fracture were unchanged when compared with sham mice. Microglial activation was quantified by assessing the extent of IBA-1 and CD11b expression within the superficial dorsal horns, ipsilateral to the fracture side. With this method, we found that dorsal horn from morphine-treated fracture mice exhibited enhanced IBA-1 and CD11b expression when compared with both sham groups and vehicle-treated fracture mice.

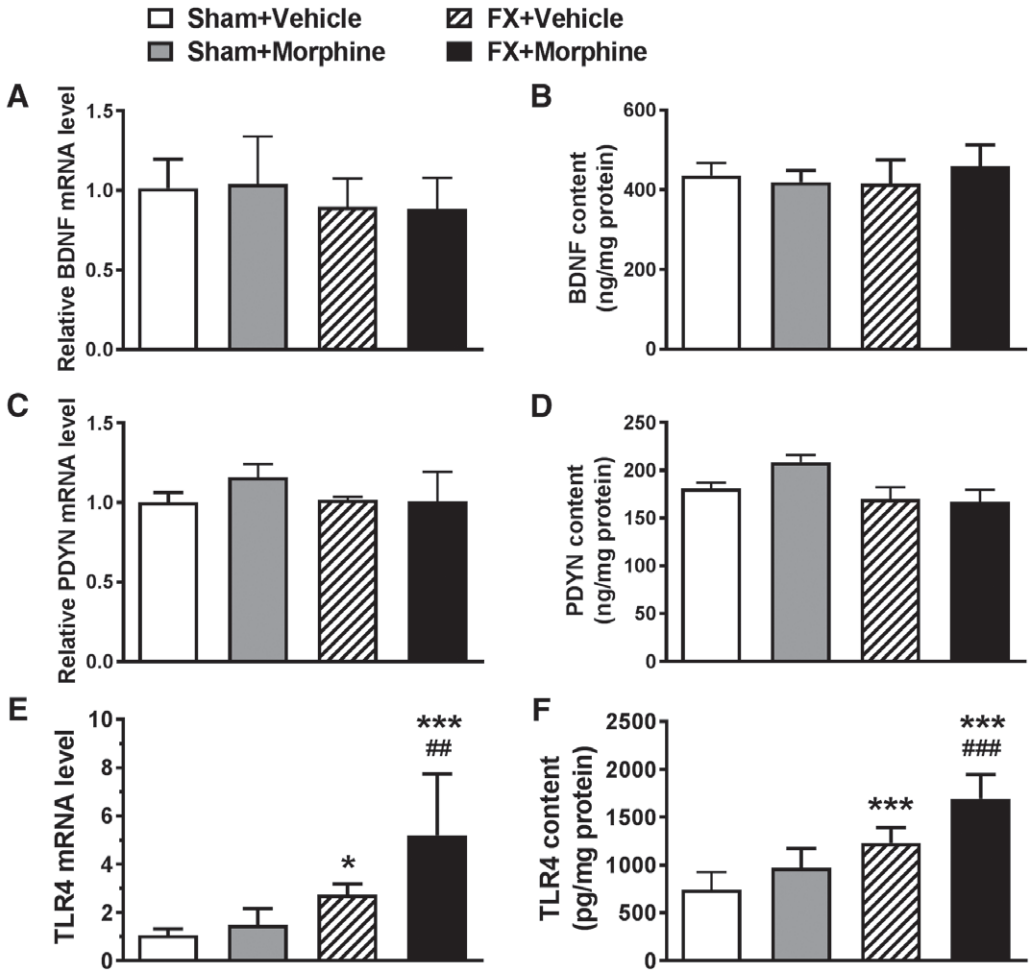

Fig. 5. Morphine administration increased spinal Toll-like receptor 4 (TLR4) but not brain-derived neurotrophic factor (BDNF) nor prodynorphin (PDYN) expression in fracture/pin (FX) mice at 2 weeks post injury. Levels of BDNF (*A* and *B*), PDYN (*C* and *D*), and TLR4 (*E* and *F*) mRNA and protein were detected using quantitative polymerase chain reaction and enzyme-linked immunosorbent assays. There were no significant differences in expression levels for either BDNF or PDYN gene between any of the groups. However, increased levels of TLR4 mRNA and protein expression were observed in vehicle-treated FX mice, and morphine treatment further dramatically enhanced this upregulation. Data were analyzed using a one-way ANOVA with Bonferroni correction test for *post hoc* contrasts. Data are expressed as mean values ± SD, N = 6 per cohort in (*A–D*), and N = 10 per cohort in (*E* and *F*). **P* < 0.05, ****P* < 0.01 FX + Vehicle or FX + Morphine *versus* Sham + Vehicle, ##*P* < 0.01, ###*P* < 0.001 FX + Morphine *versus* FX + Vehicle.

IBA-1 expression increased 2.2- and 1.5-fold in the morphine-treated fracture mice compared with sham control ($P < 0.001$, fig. 6B) and saline-treated fracture mice ($P < 0.05$, fig. 6B), respectively. Similarly, CD11b expression increased 2.5- ($P < 0.001$, fig. 6C) and 1.4-fold ($P < 0.05$, fig. 6C), respectively. Collectively, these results indicate that postfracture morphine administration enhances fracture-induced microglia activation in the lumbar dorsal horn.

Morphine Enhances Toll-like Receptor 4 Expression in Microglia 2 Weeks after Fracture

We already showed that both Toll-like receptor 4 mRNA and protein expression in the spinal cord was increased after tibial fracture and that this was further enhanced with perioperative morphine treatment. Figure 7 shows that sham control mice expressed a low level of Toll-like receptor 4 immunostaining within the superficial dorsal horn. A small but insignificant increase (1.7-fold) in Toll-like receptor 4 expression was observed in morphine-treated sham mice. In contrast, Toll-like receptor 4 expression increased significantly in the superficial dorsal horn at 2 weeks after fracture (2.8-fold increase, $P < 0.001$), which was further enhanced in the morphine-treated fracture mice (5-fold increase, $P < 0.001$). To ascertain Toll-like receptor 4 expression on microglia in these mice, spinal dorsal horn sections were incubated with an antibody directed against the microglia marker, CD11b (fig. 7A). Confocal microscopy revealed that microglia were the primary cellular source of Toll-like

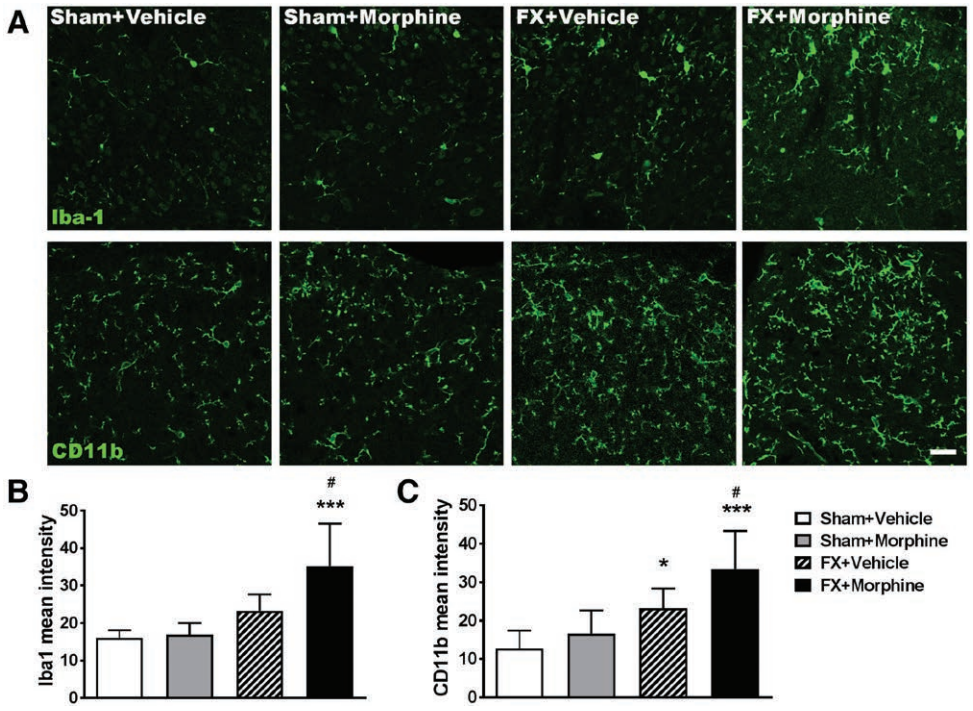

Fig. 6. Morphine administration increased spinal microglial cell activation in fracture/pin (FX) mice at 2 weeks post injury. Microglia activation in the lumbar spinal dorsal horn was assessed by immunohistochemistry using monoclonal antibody to ionized calcium-binding adapter molecule 1 (Iba-1), and polyclonal antibodies to CD11b, markers for resting and activated microglia. (*A*) Representative fluorescence photomicrographs of Iba-1 and CD11b immunostaining (*green*) in the L5 dorsal horn segment ipsilateral to fracture. (*B* and *C*) The quantification of Iba-1 and CD11b immunostaining intensity in spinal cord micrographs. Data were analyzed using a one-way ANOVA with Bonferroni correction test for *post hoc* contrasts, error bars indicate SEM. Immunostaining data are presented as mean fluorescence intensity per high powered field; *scale bar* = 25 μm. Data are expressed as mean values ± SD, N = 6 per cohort in *B*, and N = 8 per cohort in *C*. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 for FX + Vehicle or FX + Morphine *versus* Sham + Vehicle. #*P* < 0.05 for FX + Morphine *versus* FX + Vehicle.

receptor 4 in the spinal cord after fracture. Furthermore, morphine treatment after fracture further increased Toll-like receptor 4 expression in spinal microglia. Additional experiments showed that few astrocytes express Toll-like receptor 4 under these conditions (data are not shown).

Toll-like Receptor 4 Antagonism Improves Allodynia in Fracture Mice Treated with Morphine

On the basis of the behavioral and expression data, we postulated that blocking Toll-like receptor 4 activity after fracture and morphine treatment would improve pain-related behavioral outcomes. To test this hypothesis, a specific Toll-like receptor 4 inhibitor, TAK-242 (3 mg/kg, intraperitoneally), was administered once daily concurrently with morning morphine during the first 7 days after fracture. Both sham-operated and fractured mice treated with TAK-242 or the vehicle (1% dimethyl sulfoxide in water) served as controls. Figure 8A illustrates that fracture animals treated with morphine delay the nociceptive recovery as compared with the fracture mice with vehicle ($F_{(1,209)} = 60.21, P < 0.001$), administration of TAK-242 significantly attenuated allodynia in morphine-treated fracture mice ($F_{(1, 198)} = 73.36$, $P < 0.001$), but thresholds returned to those of the non–TAK-242–treated mice by 3 weeks after injury. TAK-242 treatment had no effect on nociceptive response in the sham ($F_{(1, 198)} = 0.33$, sham + TAK242 *vs.* sham + vehicle, $P = 0.57$) and the fracture mice ($F_{(1, 220)} = 0.95$, FX + TAK242 *vs.* FX + vehicle $P = 0.33$). Figure 8B demonstrates that TAK-242 treatment alleviated hind limb unweighting in the fracture mice with morphine at 2 weeks after fracture ($F_{(1, 36)} = 22.6$, $P < 0.001$) but weight-bearing was similar to fracture mice with either vehicle or TAK242 at 4 weeks after fracture. TAK-242 treatment had no effect on weight bearing in the sham ($F_{(1, 36)} = 0.16$, sham + TAK242 *vs.* sham + vehicle, $P = 0.7$) and the fracture mice ($F_{(1, 40)} = 0.001$, FX + TAK242 *vs.* FX + vehicle, $P = 0.97$). Figure 9 demonstrates that TAK-242 treatment did not improve locomotor or gait dysfunction in fracture mice treated with morphine at 2 or 4 weeks after fracture compared with relevant vehicle controls.

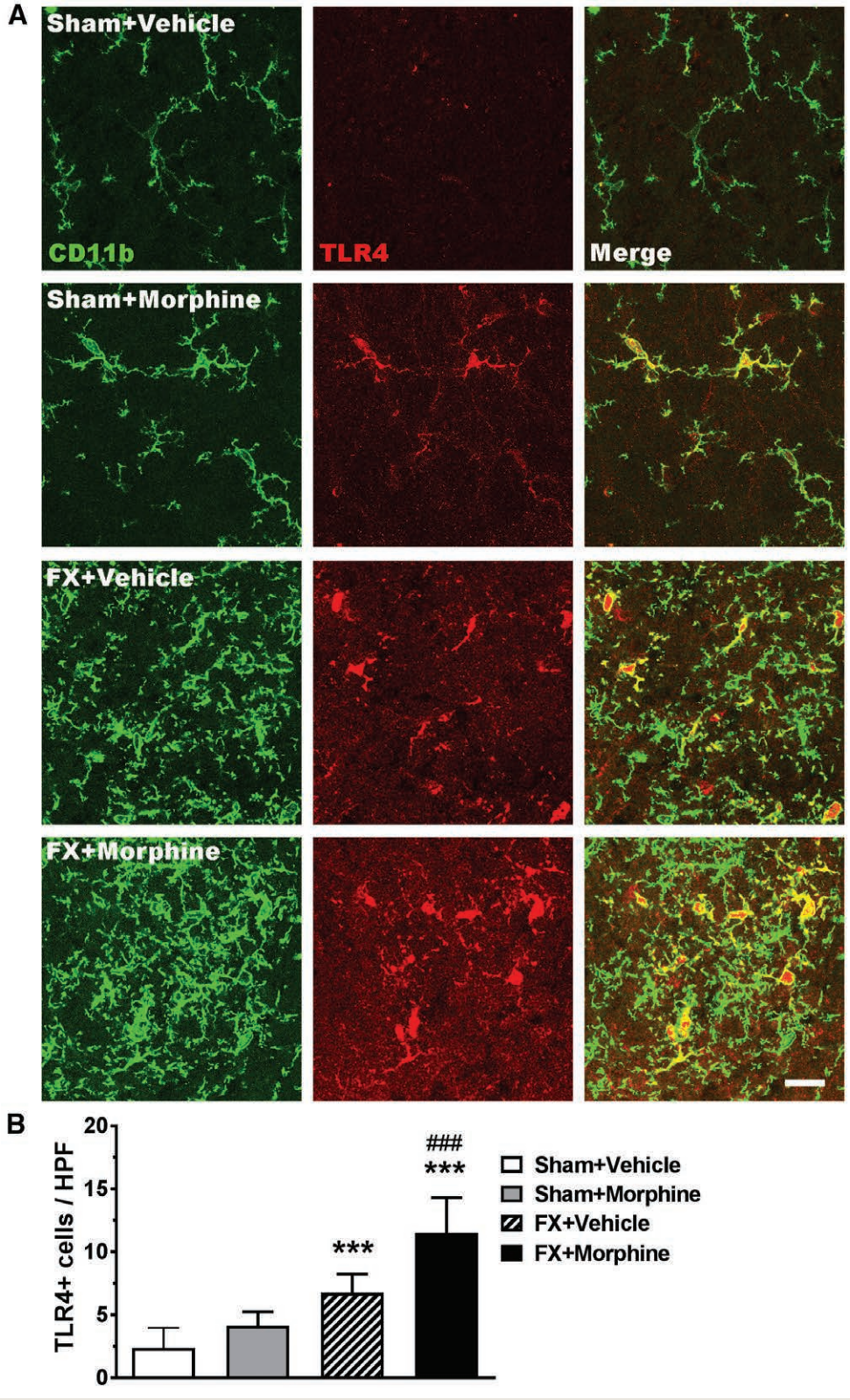

Fig. 7. Immunohistochemistry confirmed that Toll-like receptor 4 (TLR4) receptors were primarily expressed by microglial cells in the lumbar dorsal horn, and that morphine treatment amplified the post fracture/pin (FX) increase in TRL4-positive microglia at 2 weeks after injury. (*A*) Representative CD11b (*green*, microglia marker), TLR4 (*red*), and merged images for each of the treatment conditions are displayed. (*B*) Average number of TLR4-positive cells per high-power field (HPF) in the dorsal horn spinal cord. Data are expressed as mean values ± SD, N = 8 per cohort. *Scale bar* = 25 μm. ****P* < 0.001 for FX + Vehicle or FX + Morphine *versus* Sham + Vehicle, ###*P* < 0.001 for FX + Morphine *versus* FX + Vehicle.

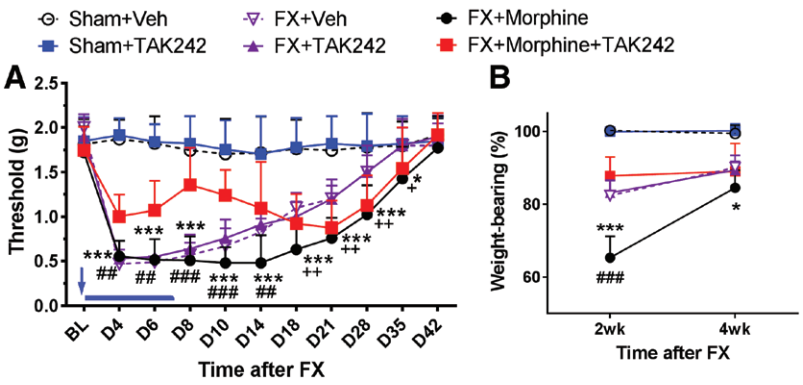

Fig. 8. Toll-like receptor 4 signaling contributes to morphine-induced nociceptive sensitization in fracture-pin (FX) mice. Mice underwent right tibia fracture and pinning, and then they were treated with morphine (*red line*) and either a Toll-like receptor 4 inhibitor (TAK-242, 3 mg/kg, intraperitoneally, *blue line*) or vehicle (Veh) for 7 days. Sham mice treated with vehicle or TAK-242 served as controls. (*A*) FX mice treated with morphine and TAK-242 exhibited less allodynia than FX mice treated morphine and vehicle. Von Frey thresholds converged at 21 days after fracture. There was no TAK-242 treatment effect on nociceptive thresholds in the sham and the fracture mice. (*B*) TAK-242 treatment alleviated hind limb unweighting at 2 weeks after injury in the morphine-treated FX mice, but this effect abated by 4 weeks after injury. *Arrow* indicates the time points at which drugs (*i.e.*, morphine or TAK-242) were injected. Data are expressed as mean values ± SD. ***$P < 0.001$ for FX + Morphine (N = 10) *versus* Sham + Vehicle (N = 10) or Sham + TAK-242 (N = 10) values, # $P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ FX + Morphine + TAK-242 (N = 10) *versus* FX + Morphine (N = 10).

Discussion

Perhaps the most widely accepted indication for opioid administration is the relief of severe acute pain. In this regard, opioids are the mainstay for pain relief after trauma and surgery. Caveats pertain to this practice, however. Acute side effects include sedation, pruritus, constipation, and respiratory depression, but there are more persistent problems as well. With the rise of the U.S. opioid epidemic, it has become apparent that physical dependence and addiction can occur if postoperative opioid use becomes chronic.[3,4] Moreover, the use of high-dose opioids at the time of trauma has been associated with poorer long-term outcomes than the use of nonopioid alternatives.[1,2] Opioid-induced hyperalgesia has long been recognized to occur in laboratory animals, in humans after high-potency opioid administration, and in humans on long-term opioid therapy for pain control or treatment of addiction.[12–14] However, little work has been done to help us understand how the perioperative use of opioids might contribute to poorer pain and functional outcomes. In the present study, we aimed to determine whether opioids administered for a limited and clinically relevant time period (7 days) in a tibia fracture orthopedic surgery model had adverse effects on postoperative recovery. Our observations suggest that a short course of opioids can, in fact, exacerbate nociceptive sensitization and functional impairment, thus prolonging recovery. Furthermore, the Toll-like receptor 4–dependent activation of spinal microglia contributes to these adverse opiate effects.

Persistent effects of opioids have been studied in animal models, but only a limited amount of work has considered the interactions of opioid administration with ongoing injury or pain. This is surprising because several types of overlap exist between the biologies of injury and chronic opioid administration, including effects on peripheral cytokine expression, protein kinase C activation, *N*-methyl-D-aspartate receptor function, spinal gene expression, activation of descending facilitation, and other responses.[12,13,44] Our own group has provided evidence that the perioperative administration of opioids slows nociceptive recovery from incision over the first several days, and that the exaggerated expression of genes involved in spinal nociceptive signal transmission, including those coding for dynorphin (prodynorphin), brain-derived neurotrophic factor, and other genes might, be involved.[10,45,46] It was felt that the interaction of surgery with morphine treatment on persistently enhanced gene expression was especially likely for the prodynorphin and brain-derived neurotrophic factor genes because enhanced spinal epigenetic histone acetylation was demonstrated to occur after morphine administration to mice that had undergone hind paw incisions.[10] Epigenetic mechanisms are thought to prolong gene expression changes and enhance nociceptive sensitization after central and peripheral injuries. It is surprisingly we found that 2 weeks after fracture and orthopedic surgery, and 1 week after completion of morphine treatment, neither of these genes showed elevated expression whereas nociceptive and functional measures remained impaired. We do note that an orthopedic injury model involving more extensive dissection was associated with longer-term changes in brain-derived neurotrophic factor expression.[47] Our observations suggest that longer-term opiate-induced sensitization in

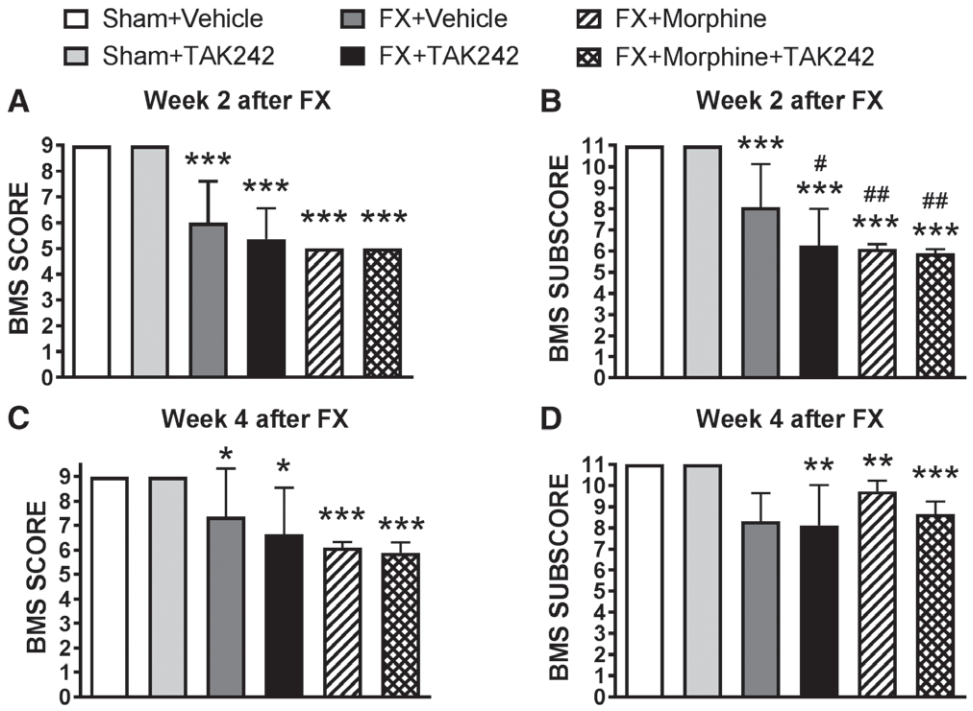

Fig. 9. Toll-like receptor 4 signaling did not contribute to morphine-induced motor dysfunction in fracture-pin (FX) mice. Basso mouse scale (BMS) scores and subscores of both sham and FX mice treated with and without morphine or the Toll-like receptor 4 antagonist TAK-242 were assessed at 2 (*A* and *B*) and 4 weeks (*C* and *D*) after fracture. Data are expressed as mean values ± SD. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 for FX + Vehicle (N = 11), FX + TAK242 (N = 11), FX + Morphine + Vehicle (N = 10) or FX + Morphine + TAK242 (N = 10) *versus* Sham + Vehicle (N = 10) or Sham + TAK242 (N = 10).

our orthopedic injury model might involve an alternate mechanism. Glial activation was considered to be one such candidate mechanism because this has been temporally and functionally associated with several types of chronic pain, including pain after tibial fracture.[48] A recent report showed that morphine treatment after nerve injury leads to glial activation and slowed nociceptive recovery.[7] Those experiments strongly implicated the participation of the microglial pattern recognition receptor Toll-like receptor 4.

Our experiments not only showed that spinal Toll-like receptor 4 was upregulated for at least 2 weeks after tibial fracture, but that morphine treatment beginning at the time of injury and continuing for 7 days greatly enhanced Toll-like receptor 4 expression at the mRNA and protein levels. Spinal expression of Toll-like receptor 4 overlapped strongly with microglia that also showed enhanced expression of the CD11b and Iba1 marker proteins after fracture and morphine treatment. The expression of Toll-like receptor 4 in the central nervous system is felt to be predominantly on microglia, although we cannot categorically exclude expression changes on other cell types. Importantly, the increase in spinal expression of Toll-like receptor 4 was associated with nociceptive sensitization, as cotreatment of mice with the Toll-like receptor 4 antagonist TAK-242 during the period of morphine administration reduced hind paw allodynia and unweighting. However, gait dysfunction was not normalized by TAK-242 treatment, perhaps indicating that analgesic effects were limited or that gait abnormalities after injury are attributable, at least in part, to nonnociceptive processes.

Numerous mechanisms have been proposed to explain how chronic opioid administration leads to opioid-induced hyperalgesia. Increased expression of peripheral algogens, enhanced spinal long-term potentiation, glial activation, augmented descending facilitation of nociceptive signaling, and other pathways have been proposed.[12,13,44] Although the present data suggest that Toll-like receptor 4 signaling in microglia contribute to the adverse opiate effects observed after fracture and surgery, alternative explanations for Toll-like receptor 4 activation exist. For example, some evidence indicates that morphine may nonstereoselectively interact with the microglial Toll-like receptor 4 receptor, leading to cytokine production and sensitization.[8,16,49] These studies demonstrated that both active (–)-morphine and non–μ-opioid receptor binding (+)-morphine are Toll-like receptor 4 agonists and can support allodynia and opioid-induced hyperalgesia in rodent models. Thus, it is possible that the Toll-like receptor 4 agonistic effects of morphine facilitated postfracture glial activation, ultimately resulting in the prolonged sensitization and functional changes observed in our studies. On the other hand, opioid-induced hyperalgesia in the absence of functional Toll-like receptor 4 receptors has also been shown to

occur.[50] It is important to note that our work did not compare male and female glial responses, although we have shown sex-linked differences in nociceptive behaviors in mice after limb injury,[51] and others have shown sex-linked differences in glial function in different pain models.[52] Ongoing work in our laboratory suggests that antinociceptive responses to glial inhibitors after tibial fracture may be sex-dependent.

An alternative mechanism for opioid-induced hyperalgesia was recently provided in which μ-opioid receptors expressed on peripheral neurons were found to be responsible for opioid-induced hyperalgesia, likely by strengthening synaptic transmission in the dorsal horn of the spinal cord.[17] These findings suggest that in our model nociceptive signaling secondary to fracture may be further enhanced by repeated morphine administration, which in turn leads to the high levels of microglial activation. These proposed mechanisms are not mutually exclusive and may both contribute. Further experimentation using peripherally restricted μ-opioid receptor antagonists, transgenic glial knockdown animals, chemically distinct opioids, and other reagents may clarify the specific mechanism.

It should be recognized that although our studies focused on nociceptive and physical function outcomes after injury, we did not attempt to assess other potentially critical types of opioid-injury interactions. For example, our data did suggest that location memory was impaired in the fracture mice receiving morphine, but not in other groups. Limb fracture has, at least in laboratory models, long-term detrimental effects on cognition and mood that opioid administration might worsen either through the enhancement of nociceptive signaling or perhaps by more directly supporting neuroinflammatory changes in the hippocampus, amygdala, and other brain centers after trauma.[29,51] Moreover, we used the archetypical opioid morphine, and we feel that results may be similar for other traditional opioids as they also cause tolerance, hyperalgesia, and glial activation. However, so-called "biased" opioids that preferentially activate specific intracellular pathways may be less likely to cause tolerance, hyperalgesia, and, perhaps, delayed surgical recovery.[53] Considering the U.S. opioid epidemic, recently declared a public health emergency, the relationship between postoperative/traumatic pain and addiction should be better elucidated. A surprisingly small number of reports have provided evidence that opioids administered in models of persistent pain can strengthen place preference for morphine and reduce the extinction of these behaviors.[54,55] Surgical models have not been well explored using place preference paradigms. Last, we are faced with the need for clinical translation of these findings: If nonopioid multi-modal analgesia is substituted for a more typical opioid-centered approach, is the longer-term quality of recovery enhanced?

In summary, we found that 1 week of morphine treatment, started at the time of fracture and surgery, exacerbated nociceptive sensitization and functional impairment through a microglial Toll-like receptor 4 mechanism. These data fit well with the existing literature and provide further support for the hypothesis that a course of opiate analgesic treatment can have long-term adverse effects in trauma and surgery patients. Future advances in understanding the interactions between opioid analgesia and nociceptive signaling may result in safer approaches to the control of severe pain.

Research Support

Support for this work was received from grant Nos. BX000881 and RX001475 from the Department of Veterans Affairs (Washington, D.C.), and grant No. R01 NS094438 from National Institutes of Health (Bethesda, Maryland).

Competing Interests

The authors declare no competing interests.

Correspondence

Address correspondence to Dr. Clark: Anesthesia Service, Veterans Affairs Palo Alto Health Care System and Stanford University, 3801 Miranda Avenue, Palo Alto, California 94304. djclark@stanford.edu.

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Headlining the Musical Backdrop to Pauline Siegel's Caesarean Section "Under Local Anesthesia"

Allen and Pauline Siegel of Chicago, Illinois, seem elated in 1941 following the successful Caesarean-section delivery of their son by obstetrician Edward L. Cornell, as performed "under local anesthesia." During the procedure, the 29-yr-old mother listened to soothing frequency-modulated (FM) radio music. Her obstetrician insisted on keeping the "pain-calmer" music "from distracting the operating staff." Seventy-seven years ago, this story made newspaper headlines nationwide. (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

George S. Bause, M.D., M.P.H., Honorary Curator and Laureate of the History of Anesthesia, Wood Library-Museum of Anesthesiology, Schaumburg, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.