Fentanyl decreases discharges of C and A nociceptors to suprathreshold mechanical stimulation in chronic inflammation

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Moshourab R, Stein C. Fentanyl decreases discharges of C and A nociceptors to suprathreshold mechanical stimulation in chronic inflammation. J Neurophysiol 108: 2827–2836, 2012. First published September 5, 2012; doi:10.1152/jn.00082.2012.—An essential component of mechanical hyperalgesia resulting from tissue injury is an enhanced excitability of nociceptive neurons, termed mechanical sensitization. Local application of opioids to inflamed rat paws attenuates mechanical hyperalgesia and reduces electrical excitability of C-fiber nociceptors in acute injury. Here, we examined the effects of the opioid receptor agonist fentanyl on the mechanical coding properties of not only C- but also A-fiber nociceptors innervating the rat hind paw in a model of chronic pain, i.e., 4 days after Freund’s complete adjuvant-induced inflammation. The peripheral mechanosensitive terminals of C-fibers (n = 143), A-fibers (n = 79), and low-threshold mechanoreceptors (n = 25) were characterized using the in vitro skin-nerve preparation from the saphenous nerve. Although mechanical activation thresholds were not changed, discharges to suprathreshold mechanical stimuli were elevated significantly in both A- and C-fiber nociceptors from inflamed tissue. In addition, the proportion of nociceptors as well as the frequency of spontaneous discharges in A (14% vs. 0%)- and C (28% vs. 8%)-fibers were increased in inflamed compared with normal tissue. Fentanyl inhibited responses to suprathreshold stimuli in a significantly higher proportion of not only C (36% vs. 7%)- but also A (41% vs. 8%)-fibers in inflamed tissue in a naloxone-reversible and concentration-dependent manner. Our results demonstrate that mechanical sensitization persists in chronic inflammation, in correlation with behavioral hyperalgesia. Opioid sensitivity of both A- and C-fibers is markedly augmented. This is consistent with an upregulation or enhanced functionality of opioid receptors located at the peripheral terminals of sensitized nociceptors.

The opioid system mediates analgesic effects both centrally and peripherally (Stein et al. 2003). In peripheral-injured tissue, sensitized nociceptors increase the expression of opioid receptors in peripheral terminals, and opioid peptide-producing immune cells migrate to the inflamed site (Brack et al. 2004; Moussa et al. 2002; Rittner et al. 2001). With progression of inflammation, centrifugal transport of opioid receptors along the axons of sensory neurons can increase steadily (Hassan et al. 1993). As a result, low doses of opioid agonists, applied locally to the inflamed area, attenuate mechanical hyperalgesia in animal models of acute as well as chronic inflammatory pain and in some clinical conditions (Stein et al. 1990b, 2003). Furthermore, stimulating immune cells to secrete their endogenous opioid peptides can result in antinociceptive effects (Schäfer et al. 1994; Stein et al. 1990a).

Inflammation causes several neurophysiological changes in the coding properties of nociceptors. These include elevated responses to suprathreshold stimuli (Andrew and Greenspan 1999), potentiation of mechanotransducers (Lechner and Lewin 2009), increased membrane excitability, and low-frequency spontaneous discharges (Djouhri et al. 2006; Xiao and Bennett 2007). For instance, in Aδ- or C-fiber nociceptors, such spontaneous discharges persist for several days after induction of Freund’s complete adjuvant (FCA) inflammation. Moreover, normally unresponsive (“silent”) Aδ- or C-fibers become sensitive to mechanical stimuli during an inflammatory process (Rukwied et al. 2008; Schmelz et al. 1994).

Only few studies have addressed the effects of opioid agonists on the coding properties in mechanosensitive terminals of cutaneous nociceptors. One study in acutely inflamed rat paws showed that morphine suppressed nociceptor discharges to suprathreshold mechanical stimuli in a dose-dependent manner in >50% of C-fibers (Wenk et al. 2006). Andrew and colleagues (1994) showed that κ- and μ-opioid agonists suppressed spontaneous discharges of C-fibers induced by short-acting ultraviolet irradiation in a concentration-dependent and naloxone-reversible manner. However, detailed examinations of opioid effects on A-fibers and of electrophysiological alterations in persistent tissue injury are lacking to date.

Here, we investigate the peripheral effects of opioids on mechanoreceptive properties of nociceptors in chronic inflammation of subcutaneous tissue using the in vitro skin-nerve preparation. We hypothesized that sensitization of C- and A-fiber nociceptors to mechanical stimuli and an opioid-induced decrease of nociceptor excitability are maintained with progression of inflammation. Because the access of opioids to their neuronal receptors is restricted by the perineurium in noninflamed tissue, we used a lipid-soluble agonist (fentanyl) with enhanced perineural permeability to en-
able comparison of normal and inflamed milieus (Antonijevic et al. 1995). We based fentanyl concentrations on previous studies of normal or acutely inflamed tissues in rats (Andreev et al. 1994; Jaffe and Rowe 1996).

METHODS

Animals and FCA-induced inflammation. All experimental procedures were approved by the Animal Care and Ethical Committees of the state authorities and are in accordance with established guidelines. A total of 81 adult male Wistar rats (200–300 g) was used in this study. FCA (150 μl; Calbiochem, San Diego, CA) was injected subcutaneously into the ventromedial area of the left hind paw of isoflurane-anesthetized rats. Rats recovered fully from anesthesia within a few minutes and were housed in standard plastic cages with soft bedding. Seventy-two to 96 h later, animals were killed for the skin-nerve preparation. In our previous behavioral studies, rats displayed increased mechanical sensitivity during that period (Stein et al. 1988b).

Skin-nerve preparation. The in vitro skin-nerve preparation was used to record responses of single primary afferents to mechanical stimulation under different pharmacological conditions (Reeh 1986; Zimmermann et al. 2009). The saphenous nerve was dissected with the skin of the hind paw attached and mounted corium-side up in an organ bath. The skin nerve was perfused at 15 ml/min with oxygen-saturated synthetic interstitial fluid (SIF) containing 108 mM NaCl, 3.5 mM KCl, 0.7 mM CaCl2, 9.5 mM sodium gluconate, 2.0 mM MgSO4, 1.7 mM NaH2PO4, 1.7 mM MgSO4, 1.7 mM NaH2PO4, 2.0 mM CaCl2, 9.5 mM sodium gluconate, 5.5 mM glucose, 7.5 mM sucrose, saturated with carbogen (95% O2–5% CO2) at pH 7.4 and 31°C.

The teased fiber technique was used for single-unit recording. The nerve was pulled gently into a separate chamber and placed on a small mirror. Under microscopy, fine strands were dissected from the nerve with sharpened watchmaker forceps. Strands were subdivided further into filaments, of which one was placed on an electrode for recording. Electrical isolation was achieved using mineral oil with the reference electrode positioned nearby in contact with SIF.

Characterization of single mechanosensitive units. The skin was stimulated with a blunted glass rod to identify mechanosensitive receptive fields of single fibers by evoked discharges. The fibers were then characterized based on von Frey thresholds, conduction velocity (CV), and response pattern to controlled mechanical displacement stimuli. The threshold to mechanical stimulation was determined with a set of calibrated von Frey filaments (Stoelting Instruments, Wood Dale, IL) with forces (in mN) and pressures (in bar) of 0.08 (0.2), 0.2 (0.5), 0.4 (0.5), 0.7 (0.6), 1.6 (0.8), 3.9 (1.6), 5.9 (1.8), 9.6 (2.3), 13.7 (2.6), 19.6 (2.7), 39.2 (3.9), 59.2 (5.1), 79 (6.1), 98 (6.6), and 148 (8.3). The monofilament tip area ranged from 0.064 to 0.114 mm². To determine CVs, a sharp tungsten electrode was lowered onto the most sensitive spot in the receptive field to deliver suprathreshold electrical current pulses of 50, 150, or 500 μs. The distance and latency of action potentials in each isolated unit were recorded, yielding CVs typical for C (<1.6 m/s), Aδ (1.6–13 m/s), and Aβ (>13 m/s)-fibers (Zimmermann et al. 2009).

Spontaneous activity. Spontaneous activity was recorded in three inflamed and three normal paws. Each teased nerve filament placed on the recording electrode contained, on average, four to seven single mechanosensitive units, which were isolated by optical stimulation. To calculate the percentage of spontaneously active units, we counted the total number of distinct waveforms present in each filament.

Mechanical stimulation of single mechanosensitive units. Mechanical stimuli were delivered by a stainless-steel rod with a flat circular contact area of 0.5 mm² attached to a computer-driven nanomotor (Kleindiek, Reutlingen, Germany), which was maneuvered onto a spot within the receptive field where the most reliable responses could be obtained with a von Frey filament (Milenkovic et al. 2008). The probe was advanced perpendicularly and moved in steps whose amplitude was reduced systematically so that the smallest possible stimulus reliably evoked at least one spike. The unit was then confronted with an ascending series of preprogrammed displacement stimuli. The standard ramp speed was 2.350 μm/s. Displacement stimuli of 50, 100, 200, and 400 μm duration were applied at regular intervals of 30 s. The total number of evoked spikes and mechanical latency (time between onset of the ramp and first recorded spike corrected for conduction delay, electrical latency; see Fig. 7A) was recorded. The forces exerted by the nanomotor on the skin were not measured directly, but in a previous study using an identical stimulation technique, we demonstrated a linear relationship between increasing displacements (50–500 μm) and force (Milenkovic et al. 2008).

Classification of mechanosensitive units. The sampled mechanosensitive units were divided into three groups: C-mechanonociceptors (CM), A-fiber mechanonociceptors (AM), and low-threshold slowly adapting Aβ-mechanoreceptors (LTM). Low-threshold CM, characterized by a von Frey mechanical threshold <6 mN, and afterdischarges to mechanical stimulus removal are known to have nociceptive tactile function and were therefore excluded from the CM sample (Leem et al. 1993; Lynn and Carpenter 1982; Olausson et al. 2010). AM mainly consisted of Aδ-mechanonociceptors and fibers that conducted in the Aβ CV range. These Aβ-fibers were functionally classified as nociceptors, according to the following criteria: 1) they did not discharge during the ramp phase of the 50-μm stimulus, and 2) they increased their discharge to increasing stimulus intensities (Djouhri and Lawson 2004; Milenkovic et al. 2008; Treede et al. 1998). Rapidly adapting low-threshold Aβ- and Aδ-mechanoreceptors (D-hairs) were not studied. Receptive field sizes excited by von Frey hairs of >6.1 bars (80 mN hair with 0.406 mm diameter) (Meyer et al. 1991) were labeled as very-high threshold or mechanically insensitive units and were not subjected to the mechanical stimulation protocol.

Fentanyl application. All mechanosensitive receptive fields in inflamed and normal tissue were subjected to a first (stim 1) series of graded mechanical stimuli under SIF exposure that served as baseline. Five minutes later, vehicle (SIF) or drug was applied. Stock solutions of fentanyl citrate (Sigma-Aldrich, St. Louis, MO) were diluted with SIF (pH 7.4). Drug solutions were applied directly to the corium through a small metal ring (10 mm inner diameter) for separation from SIF buffer. In this manner, the receptive field was exposed to 100 μl oxygen-saturated SIF with 50 nM, 1 μM, or 25 μM fentanyl for 3 min before the second (stim 2) series commenced (Fig. 1). Thus the total time of exposure to fentanyl was ~5 min.

Selection of fentanyl-sensitive nociceptors. Nociceptor responses to successive mechanical stimulations of the same magnitude can vary substantially (Slugg et al. 2000). To distinguish a normal change in nociceptor response to repeated stimulation from a potential drug effect, we first determined the upper and lower range of changes from baseline in control and inflamed skin. In pilot experiments using 9 AM and 10 CM, we determined the variability of responses (number of spikes/stimulus) between stim 1 and stim 2 in real-time using the LabChart software (with spike histogram extension). The normal variability of responses under SIF exposure (vehicle) was defined as 2 SD around the mean (AM: 61–150%; CM: 73–131%). We compared the sum of the spikes discharged with the 200- and 400-μm stimuli and expressed the variability for each unit as the ratio between responses to stim 1 and stim 2 (as percentage). The mean response rates did not differ significantly between series. The normal variability of responses under SIF exposure was defined as 2 SD around the mean (AM: 106.2 ± 22.3%; CM: 102.3 ± 14.4%; mean ± SD).

Units were categorized as fentanyl-sensitive if their response decreased by >2 SD below the mean (i.e., <61% for AM or <73% for CM, respectively). With the use of this criterion, drug-induced effects were distinguished from normal variability due to repeated stimulation, similar to a previous study (Wenk et al. 2006). After a >10-min washout period, fentanyl-sensitive units were tested for naltrexone reversibility. Baseline mechanical stimulation was performed, and 5 min later,
naloxone (in equimolar concentration) was applied for 2 min, followed by fentanyl for 3 min. Thereafter, another stimulation series commenced. Naloxone alone (25 μM) was applied for 2 min, followed by a 2nd series of stimulation commenced.

**Fig. 1.** Scheme of the experimental protocol. A: an example trace of an A-fiber mechanonociceptor (AM) is shown. A computer-controlled nanomotor delivered 10-s mechanical displacement stimuli from 50 μm to 400 μm at 30-s intervals to receptive fields. Application of fentanyl was started 5 min after the 1st stimulation series and 3 min before a 2nd series of stimulation commenced. B: the responses of each single fiber were plotted, and the areas under the curve (AUC) of the 1st (stim 1) and 2nd (stim 2) stimulation series were calculated. C: the AUC ratio quantified fentanyl-induced changes in each unit. CV, conduction velocity; SIF, synthetic interstitial fluid.

**RESULTS**

Conduction velocities and von Frey thresholds are unaltered in inflammation. We recorded from 247 single units (79 control and 168 inflamed) isolated from the saphenous nerves of 75 animals (53 rats with inflamed hind paw and 22 control rats). Among the 168 units from inflamed paws were 99 CM, 54 AM, and 15 LTM. Among the 79 units from normal paws were 44 CM, 25 AM, and 10 LTM (Table 1). The mean CVs of each fiber type were not statistically different between normal and inflamed paws. Among the 168 units from inflamed paws was 99 CM, 54 AM, and 15 LTM (Table 1). The mean CVs of each fiber type were not statistically different between normal and inflamed paws (P > 0.05, t-test). The median monofilament thresholds of each fiber type were not significantly different between normal and inflamed paws (P > 0.05, for AM, LTM, and CM, Mann-Whitney U-test).

**Nociceptors from inflamed paws have increased spontaneous activity.** The prevalence and magnitude of spontaneous activity in both C- and A-fibers were increased in inflamed paws. We found 29% (26/91) of CM and 14% (eight of 56) of AM with an unprovoked, ongoing activity in inflamed vs. 8% (six of 80) CM and 0% (zero of 58) AM in normal paws (P < 0.005, Fischer’s exact test in both cases). The average discharge frequency of CM over a 60-s recording period was significantly higher in inflamed (0.85 ± 0.16 spikes/s) compared with normal (0.54 ± 0.37 spikes/s; P = 0.038, Mann-Whitney U-test) paws. The AM discharge frequency in inflamed paws was 0.94 ± 0.32 spikes/s. We also found six...
Table 1. Physiological properties of mechanosensitive primary afferents from control and inflamed paws

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<td>LTM A-Fibers</td>
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<td>AM A-Fibers</td>
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<td>CM A-Fibers</td>
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Conduction velocity (CV) are means ± SD. von Frey thresholds (vFT) are median with interquartile range (quartiles 1–3). No significant differences were found between the two conditions. LTM, low-threshold mechanoreceptors; AM, A-fiber mechanonociceptors; CM, C-fiber mechanonociceptors.

(23%) units with unprovoked, ongoing burst discharges of two or more spikes (in addition to single-spike irregular firing) with an average frequency of 1.85 ± 0.46 spikes/s in inflamed (but none in normal) tissue (Fig. 2, A and B). In five C-fibers from inflamed (but none from normal) tissue, a brief response to the mechanical search probe was followed by prolonged (3–6 min) burst-like activity, which transformed to regular, single-spike firing, diminishing within minutes (Fig. 2C).

Nociceptors in inflamed tissue are sensitized to suprathreshold mechanical stimulation. As demonstrated by the number of action potentials evoked by suprathreshold mechanical stimuli, both CM and AM in inflamed paws exhibited significant sensitization compared with noninflamed control preparations (CM: control, n = 44; inflamed, n = 54, P = 0.0295; AM: control, n = 25; inflamed, n = 32, P = 0.0119, two-way RM-ANOVA; Fig. 3, B and D). The maximal mechanical stimulation (400 μm displacement) elicited significantly more spikes in CM of inflamed compared with noninflamed paws (CM: 89 ± 9 vs. 62 ± 7 spikes/stimulus ± SE; Bonferroni post hoc tests, P < 0.05; Fig. 3B). In inflamed tissue, significant sensitization of AM was evident at even lower mechanical displacements of 200 μm (AM: 148 ± 29 vs. 95 ± 11 spikes/stimulus ± SE; Bonferroni post hoc tests, P < 0.05; Fig. 3D). AB nociceptors (four of 25 noninflamed, five of 32 inflamed; CV > 13 m/s) were included in the analysis of AM, since their stimulus-response plots did not differ significantly from Aδ-fibers in both inflamed and normal paws. The response latencies were not different between inflamed and control paws (AM: 57 ± 6 ms vs. 53 ± 7 ms; CM: 93 ± 11 ms vs. 75 ± 7 ms; means ± SE; P > 0.05, unpaired t-tests). The stimulus response properties of LTM did not differ significantly between inflamed and noninflamed paws (data not shown). Thus FCA inflammation caused marked mechanosensitization, specifically in CM and AM nociceptors but not in LTM.

Fentanyl does not modulate discharges of nociceptor population-inervating normal tissue. Twenty-six nociceptors (14 CM and 12 AM) from control paws were treated with 25 μM fentanyl. The AUC for baseline vehicle or fentanyl exposure was calculated to derive the AUC vehicle ratio (AUC vehicle/AUC baseline) and AUC fentanyl ratio (AUC fentanyl/AUC baseline), respectively. In control tissue, the mean AUC vehicle ratio was 1.03 ± 0.14 for CM (mean ± SD; n = 8) and 0.96 ± 0.11 for AM (mean ± SD; n = 7). The lower cutoff values for selecting fentanyl-sensitive units defined in METHODS (73% for CM; 61% for AM) were in agreement with the cutoff values based on the AUC vehicle ratio calculation (0.75 for CM, equivalent to 75% of baseline AUC; 0.74 for AM, equivalent to 74% of baseline AUC). Thus units that showed a drug-evoked inhibition by at least 73% for CM and 61% for AM were classified as fentanyl sensitive.

Fentanyl decreased the AUC ratio of one of 14 CM and one of 12 AM below the respective cutoff values. After washout and return of baseline response, naloxone prevented the second decrease of the AUC ratio in the fentanyl-sensitive AM and CM units (Fig. 4, A and B). In one C-fiber, fentanyl evoked an excitation to 141% of baseline value. Overall, fentanyl produced neither a significant decrease in AUC ratio nor a decrease in inhibition in the CM and AM nociceptor population compared with vehicle (P > 0.05, Mann Whitney U-test; Fig. 4, C and D).

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TO QUANTIFY THE EFFECTS OF FENTANYL ON THE WHOLE POPULATION OF AM AND CM NOCICEPTORS IN INFLAMED SKIN, THE MEAN AUC RATIOS (AUCfentanyl/AUCbaseline) FOR EACH GROUP AND CONDITION WERE COMPARED WITH CONTROL (VEHICLE; Fig. 5, A AND B). UNDER 25 μM FENTANYL, A SIGNIFICANT DECREASE IN MEAN AUC RATIO WAS OBSERVED (0.98 ± 0.17 vs. 1.00 ± 0.00, P = 0.007, MAN-WHITNEY U-TEST; Fig. 5A) AND AM (0.88 ± 0.07 vs. 1.00 ± 0.05, P = 0.046, MAN-WHITNEY U-TEST; Fig. 5B) COMPARED WITH VEHICLE (1.09 ± 0.21 AND 1.19 ± 0.31, RESPECTIVELY).

CONCENTRATION-RESPONSE RELATIONSHIPS OF FENTANYL WERE DERIVED FROM 55 CM AND 29 AM NOCICEPTORS (Fig. 5, C AND D). CM RESPONSES WERE INHIBITED BY 2.9% AT 50 nM UP TO 20% AT 25 μM FENTANYL. COMPARED WITH VEHICLE (−9.4 ± 6.6%, n = 11), THIS INHIBITION WAS STATISTICALLY SIGNIFICANT AT 25 μM (10.6 ± 6.7%, n = 28; MANN-WHITNEY U-TEST, P = 0.025; Fig. 5C). FENTANYL INHIBITED AM FIBERS BY 33% AND 30.7% AT 1 AND 25 μM CONCENTRATIONS, RESPECTIVELY. FROM CURVE FITTING, WE ESTIMATED AN IC50 VALUE OF 565 nM FOR CM AND 930 nM FOR AM (Fig. 5, D). Fentanyl modulation of nociceptors is enhanced in inflamed tissue.

Fentanyl modulation of nociceptors is enhanced in inflamed tissue. The terminals of CM and AM nociceptors from inflamed paws were treated with fentanyl at concentrations of 50 nM, 1 μM, and 25 μM. According to our definition of fentanyl sensitivity (see METHODS), fentanyl inhibited zero of 10 CM at 50 nM, six of 19 CM at 1 μM, and 10/28 CM at 25 μM concentration (Fig. 5A). In inflamed tissue, fentanyl inhibited a significantly higher proportion of CM (six of 19 at 1 μM; 10/28 at 25 μM) compared with normal tissue (one of 14 at 25 μM; P < 0.05, CH2-test). Similarly, fentanyl inhibited significantly more AM (two of six at 1 μM; seven of 17 at 25 μM; Fig. 5B) in inflamed than in normal tissue (one of 12 at 25 μM; P < 0.05, CH2-test). Fentanyl evoked excitation of one of 10 CM at 50 nM, three of 19 CM at 1 μM, and five of 28 CM at 25 μM and in two of 17 AM at 25 μM concentration. Vehicle excited one of 10 CM and one of eight AM compared with baseline.

To quantify the effects of fentanyl on the whole population of AM and CM nociceptors in inflamed skin, the mean AUC ratios (AUCfentanyl/AUCbaseline) for each group and condition were compared with control (vehicle; Fig. 5, A and B). Under 25 μM fentanyl, a significant decrease in mean AUC ratio was observed both in CM (0.89 ± 0.35; P = 0.026, Mann-Whitney U-test; Fig. 5A) and AM (0.88 ± 0.57; P = 0.043, Mann-Whitney U-test; Fig. 5B) compared with vehicle (1.09 ± 0.21 and 1.19 ± 0.31, respectively).

Concentration-response relationships of fentanyl were derived from 55 CM and 29 AM nociceptors (Fig. 5, C and D). CM responses were inhibited by 2.9% at 50 nM up to 20% at 25 μM fentanyl. Compared with vehicle (−9.4 ± 6.6%, n = 11), this inhibition was statistically significant at 25 μM (10.6 ± 6.7%, n = 28; Mann-Whitney U-test, P = 0.025; Fig. 5C). Fentanyl inhibited AM fibers by 33% and 30.7% at 1 and 25 μM concentrations, respectively. From curve fitting, we estimated an IC50 value of 565 nM for CM and 930 nM for AM (Fig. 5, D). Fentanyl modulation of nociceptors is enhanced in inflamed tissue.

Naloxone prevents fentanyl-induced inhibition. To determine whether naloxone blocks the inhibition in fentanyl-sensitive CM (n = 16) and AM (n = 9) fibers, we first recorded another baseline (baseline 2) response after a washout period of at least 10 min. Pretreatment with naloxone abolished the inhibition induced by an equimolar fentanyl concentration in both CM and AM fibers (mean AUC values were compared using the Kruskal-Wallis test, followed by Dunn’s post hoc test; Fig. 6, A and B). Naloxone alone (25 μM) had no significant effect on AM (n = 3) and CM (n = 6) nociceptors from inflamed paws.

Modulation of response latencies. The response latencies to the 400-μm mechanical stimulus were not changed by fentanyl in any CM, LTM, or fentanyl-sensitive AM in inflamed tissue (P > 0.05, Kruskal-Wallis test; Fig. 7B). In AM fibers whose discharge was not inhibited by fentanyl, mean latencies were decreased significantly compared with baseline (55.7 ± 19.4 ms vs. 37.1 ± 13.4 ms, n = 20; P = 0.004, Mann Whitney U-test; Fig. 7C). There were no significant changes in response latencies of any CM or AM treated with fentanyl in normal tissue (P > 0.05, Mann Whitney U-test; data not shown).

DISCUSSION

Our study demonstrates that the opioid receptor agonist fentanyl modulates the mechanical coding properties of both
A-fiber nociceptors. These data provide electrophysiological evidence that nociceptor sensitization and opioid sensitivity are not only detectable in acute injury but are also maintained in persistent tissue inflammation.

Neurophysiological correlates of mechanical hyperalgesia. Subcutaneous FCA-induced inflammation results in mechanical hyperalgesia, which starts as early as 4 h, reaches a maximum during the first 3 days, and can last up to 14 days (Newbold 1963; Stein et al. 1988a). To demonstrate mechanical sensitization of nociceptors in an electrophysiological setting, at least 50 µg weight or 100 µl distension volume is needed (Andrew and Greenspan 1999; Du et al. 2003; Wenk et al. 2006). Although lower dosages of FCA can induce thermal without mechanical hyperalgesia (Fraser et al. 2000; Iadarola et al. 1988), the latter is considered more relevant in clinical conditions (Mantyh et al. 2006; Schaible et al. 2009). A consistent feature following inflammation is the increased number of nociceptors with spontaneous activity. Previous studies reported a large proportion (~25%) of A- and C-fiber nociceptors with unprovoked, low-frequency, ongoing activity in FCA paw inflammation. This was maximal by 2 days, persisted up to 7 days, and diminished to control levels by 14 days (Xiao and Bennett 2007). We found similar rates of spontaneous activity in A- and C-fibers. Such ongoing activity has been correlated with persistent pain sensations in inflammatory and neuropathic pain models (Djouhri et al. 2006) and is thought to be evoked by inflammatory mediators and tissue acidosis (Steen et al. 1996).

It has been difficult to demonstrate convincingly sensitization of nociceptors in terms of altered mechanosensitivity following inflammation. Two main parameters are usually studied: mechanical activation thresholds and responses to suprathreshold stimulation. In studies using short-lived inflammatory stimuli, no changes in mechanical thresholds could be detected (e.g., capsaicin, mustard oil, carrageenan) (Baumann et al. 1991; Handwerker et al. 1987; Milenkovic et al. 2008; Reeh et al. 1987). Skin incision or inflammatory models yielded controversial findings concerning activation thresholds (Andrew and Greenspan 1999; Banik and Brennan 2008; Hämläinen et al. 2002; Pogatzki et al. 2002; Wenk et al. 2006). Few studies could detect modest changes in mechanical threshold after an inflammatory insult with carrageenan or FCA (Kocher et al. 1987; Wenk et al. 2006). With the use of electrical search techniques, Wenk et al. (2006) found decreased proportions of very high-threshold nociceptors and decreased mechanical thresholds in FCA inflammation. The failure to replicate this observation in our study might be due to a sampling bias inherent in our mechanical search technique. As a consequence, our samples might contain different amounts of mechanically insensitive or very high-threshold nociceptors (greater than six bars) (Meyer et al. 1991). The proportion of mechanically insensitive afferents (MIA) is ~20% in rat skin (Handwerker et al. 1991; Kress et al. 1992). However, MIA can acquire mechanosensitivity after stimulation with capsaicin or mustard oil (Kress et al. 1992; Schmelz et al. 2000; Schmidt et al. 1995, 2000). We did not distinguish between sensitized MIA and mechanonociceptors in our model of inflammation, although we did note that our A-fiber nociceptors from inflamed skin had lower CVs that is in agreement with the observation from Handwerker et al. (1991) and Treede et al. (1998) that the majority of MIA are slowly conducting.

In our study, sensitized A- and C-fiber nociceptors exhibited increased peak firing using forces considerably above mechanical activation thresholds, in line with previous studies (Andrew and Greenspan 1999). Both the diameter of the stimulating probe and stimulus intensity are important variables characterizing nociceptor sensitization (Garell et al. 1996). For instance, applying the same mechanical stimulus of 90 g to inflamed skin, increased responses were found with probes of 0.1 mm² and 1 mm² in A-fiber nociceptors but only with a 0.1-mm² probe in C-fibers.
Wenk et al. (2006) applied mechanical stimuli of 3.3 (34 g/mm²) and 5.5 bars (56 g/mm²) with a probe of 4.9 mm² and did not observe significant differences in discharge rates between nociceptors from control and inflamed rat tissue. We used a probe of 0.5 mm² and demonstrated a robust increase of responses in both A- and C-fiber nociceptors in inflamed tissue. Thus both chronicity of the inflammation and stimulus characteristics are important variables for comparison of different studies. It seems that mechanical thresholds of nociceptive afferents can change within seconds, depending on the context of stimulation (Steen et al. 1992), and new patterns of discharge to mechanical stimuli (e.g., bursting), in addition to increased firing, can emerge. However, a spike-train analysis of nociceptor responses would be beyond the scope of our study.

**Opioid modulation of nociceptors.** Opioid receptors (µ, δ, and κ) are differentially expressed by small- and large-diameter dorsal root ganglion (DRG) neurons (Busch-Dienstfertig and Stein 2010; Búzás and Cox 1997; Coggeshall et al. 1997; Maekawa et al. 1994; Wang and Wessendorf 2001). Small-diameter Aδ- and C-fibers are the predominant types that transmit nociceptive signals to the spinal cord dorsal horn under physiological conditions. The majority of small DRG neurons is peptidergic, and 50% expresses µ-opioid receptors under normal conditions (Li et al. 1998; Silbert et al. 2003; Wang et al. 2010). Particularly in inflamed tissue, opioid receptor agonists applied peripherally reduce behavioral mechanical and thermal hyperalgesia (Stein 1995). These antinociceptive effects are mediated by opioid receptors expressed at the peripheral terminals of nociceptors. In one study using the skin-nerve preparation, ongoing activity from inflammation triggered by short-lasting UV radiation was suppressed by opioid agonists in concentrations up to 20 µM (Andreev et al. 1994). During the development of persistent FCA inflammation,
tion, there is an increase in neurons expressing opioid receptors and in receptors/neuron and a time-dependent upregulation of opioid receptor mRNA in the DRG with surges of /H9262-opioid receptor expression at 2 and 96 h (Mousa et al. 2007; Puehler et al. 2004, 2006). Axonal transport of opioid receptors to peripheral terminals is enhanced, and this has been attributed to inflammatory mediators (cytokines and NGF), as well as electrical conduction (Puehler et al. 2004, 2006). In addition, the disruption of the perineurial barrier might facilitate the access of exogenous opioid agonists to neuronal opioid receptors (Antonijevic et al. 1995; Rittner et al. 2009). In contrast, opioids applied along the axon in the absence of inflammation apparently fail to produce antinociceptive effects or conduction block (Grant et al. 2001; Jaffe and Rowe 1996; Picard et al. 1997; Senami et al. 1986; Yuge et al. 1985). For instance, fentanyl in concentrations up to 3 μM did not block conduction in dorsal roots of normal rats in vitro (Jaffe and Rowe 1996). Furthermore, whereas there is controversy as to which nociceptor fiber types express opioid receptors under normal conditions (Scherrer et al. 2009; Wang et al. 2010), such studies

Fig. 6. Naloxone prevents fentanyl-induced inhibition in inflamed paws. Bar graphs show AUC (means ± SD) calculated from stimulus-response plots of single fentanyl-sensitive CM and AM. Fentanyl significantly reduced the mean AUC of CM (A) and AM (B). After a washout period of >10 min, baseline stimulation demonstrates recovery from fentanyl inhibition. Pretreatment with equimolar concentrations of naloxone prevented reduction of mean AUC. (Kruskal-Wallis test with Dunn’s post hoc test; CM, n = 16; AM, n = 9; *P < 0.05; **P < 0.01).

Fig. 7. Latencies of discharges to mechanical stimuli in C- and A-fibers. A: an example describing measurement of latency after initiation of mechanical stimulation. e.lat, electrical latency; m. lat., mechanical latency. B: no significant changes in latencies of fentanyl-sensitive CM and AM fibers under fentanyl and after pretreatment with naloxone (Kruskal-Wallis test with Dunn’s post hoc test). C: latencies of fentanyl-insensitive AM but not of CM or low-threshold slowly adapting Aβ-mechanoreceptors (LTM) were significantly altered after addition of fentanyl in inflamed tissue (Mann-Whitney U-test, *P < 0.05).
have not been performed under inflammatory conditions. Thus it is important to take into account all of these neuroplastic changes when investigating peripheral antinociceptive effects of opioids. Our data demonstrate that both C- and Aδ-fibers express functional opioid receptors in inflamed and in normal tissue and that the proportion of both opioid-sensitive fiber types increases drastically during inflammation.

We also analyzed the latency between initiation of mechanical stimulation and generation of the first action potential, which reflects the process of mechanotransduction. This process is dependent on the interaction of mechanically gated and voltage-gated ion channels. In the fentanyl-sensitive subpopulations of C and A nociceptors, no significant changes in latencies were observed, which suggests that opioids do not alter the mechanotransduction process. Surprisingly, under fentanyl, decreased latencies in the “fentanyl-insensitive” subpopulation of A nociceptors in inflamed tissue were observed. Possible explanations include an increased excitability caused by the first stimulation or changes in tissue compliance of inflamed tissue, which can confound our measurements. These issues need to be elaborated in future studies.

A surgical skin incision with tissue inflammation can lead to mechanical hyperalgesia for several days in rodents (Brennan et al. 1996) and humans (Wilder-Smith et al. 2010). Sensitization of A-fiber nociceptors seems to be more prominent than C-fibers (Pogatzki et al. 2002). Whether opioids in this setting reduce A-fiber discharges and thereby contribute to analgesia needs to be investigated. There are currently no human studies examining nociceptor sensitization in inflammation.

In summary, we found that the activation of opioid receptors on peripheral neurons innervating chronically inflamed rat paws can suppress single nociceptor discharges to intense mechanical stimuli. These data extend previous studies in that we now show that a substantial proportion of sensitized A-fiber nociceptors, in addition to C-fibers, is inhibited by opioid agonists. These data provide electrophysiological evidence that nociceptor sensitization and opioid sensitivity are not only detectable in acute injury but are also maintained in persistent tissue inflammation. This provides a basis for therapeutic opioid modulation of chronic mechanical hyperalgesia in inflammatory conditions such as arthritis, fibromyalgia, and postoperative or cancer pain. Future studies will have to investigate what particular conditions might optimize and maximally augment this opioid receptor modulation of nociceptor sensitization in chronic inflammation.

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AUTHOR CONTRIBUTIONS
Author contributions: R.M. and C.S. conception and design of research; R.M. performed experiments; R.M. analyzed data; R.M. interpreted results of experiments; R.M. prepared figures; R.M. drafted manuscript; C.S. edited and revised manuscript; R.M. and C.S. approved final version of manuscript.

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