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

Authors: Rabih Moshourab, Christoph Stein
Affiliation: Department of Anesthesiology and Critical Care Medicine, Freie Universität Berlin, Charité Campus Benjamin Franklin, Hindenburgdamm 30, 12203 Berlin, Germany.
Contact: e-mail: rabih.moshourab@charite.de
Running head: opioids inhibit C and A nociceptors in chronic inflammation
Keywords: nociceptor, inflammation, opioids, peripheral
Abstract

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 hindpaw 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 significantly elevated 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 to normal tissue. Fentanyl inhibited responses to suprathreshold stimuli in a significantly higher proportion of not only C- (36 % vs. 7 %) but also A-fibers (41 % vs. 8 %) in inflamed tissue in a naloxone-reversible and concentration-dependent manner. Our results demonstrate that mechanical sensitization persists in chronic inflammation, in correlation with behavioural 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.
1. Introduction

Mechanical hyperalgesia develops after tissue inflammation and is defined as an augmented reaction to normally painful mechanical stimuli. In patients suffering from acute or chronic pain, this is one of the most common problems requiring therapeutic intervention (Mantyh, 2006; Schaible et al., 2009). Underlying neuroplastic changes include enhanced responsiveness of second-order neurons in the spinal cord to input from primary afferent neurons activated by noxious stimuli (A- and C-nociceptors), a phenomenon referred to as central sensitization (Woolf and Salter, 2000). In addition, increased nociceptor excitability, known as peripheral sensitization, is thought to play a role in skin, muscle, and viscera (Andrew and Greenspan, 1999; Gebhart et al., 2000; Lewin and Moshourab, 2004; Gold and Gebhart, 2010).

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 (Rittner et al., 2001; Mousa et al., 2002; Brack et al., 2004). With progression of inflammation, centrifugal transport of opioid receptors along the axons of sensory neurons can steadily increase (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 (Stein et al., 1990a; Schäfer et al., 1994).

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 (Schmelz et al., 1994; Rukwied et al., 2008).

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 more than 50% of C-fibers (Wenk et al., 2006). Andreev and colleagues showed that kappa- and mu-opioid agonists suppressed spontaneous discharges of C-fibers induced by short-acting ultraviolet irradiation in a concentration-dependent and naloxone-reversible manner (Andreev et al., 1994). 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 opioid-induced decrease of nociceptor excitability is 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 perineurial permeability to enable comparison of normal and inflamed milieus (Antonijevic et al. 1995). We based fentanyl concentrations on previous studies on normal or acutely inflamed tissues in rats (Andreev et al. 1994; Jaffe et al. 1996).
2. Methods

Animals and Freund's complete adjuvant-induced inflammation

All experimental procedures were approved by the animal care and ethical committees of the State authorities and are in accordance to established guidelines. A total of 81 adult male wistar rats (200-300 g) were used in this study. FCA (Calbiochem Corp.; 150 µl) was injected subcutaneously into the ventromedial area of the left hindpaw of isoflurane-anesthetized rats. Rats recovered fully from anesthesia within a few min and were housed in standard plastic cages with soft bedding. Seventy-two to 96 h later animals were sacrificed 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 hindpaw 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 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 gently pulled 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 further subdivided 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) 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), 148 (8.3). The monofilament tip area ranged from 0.064-0.114 mm$^2$. 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-fibers (> 13 m/s) (Zimmermann et al., 2009).

**Spontaneous activity**

Spontaneous activity was recorded in 3 inflamed and 3 normal paws. Each teased nerve filament placed on the recording electrode contained on average 4-7 single units. Any unit firing at least more than 1 spike/min was considered spontaneously active. We characterized the unit based on its waveform and CV. 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) 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 systematically reduced so that the smallest possible stimulus reliably evoked at least one spike. The unit was then confronted with an ascending series of pre-programmed displacement stimuli. The standard ramp speed was 2350 µm/s. Displacement stimuli of 50, 100, 200 and 400 µm of 10 s 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) were recorded. The forces exerted by the nanomotor on the skin were not measured directly but in a previous study employing an identical stimulation technique, we demonstrated a linear relationship between increasing displacements (50 to 500 µm) and force (Milenkovic et al., 2008).

Classification of mechanosensitive units

The sampled mechanosensitive units were divided into three groups: C-mechanoreceptors (CM), A-fiber mechanoreceptors (AM) and low threshold slowly-adapting Aβ-mechanoreceptors (LTM). Low threshold C-mechanoreceptors characterized by von Frey mechanical threshold < 6 mN and afterdischarges to mechanical stimulus removal are known to have non-nociceptive tactile function and were therefore excluded from the CM sample (Lynn and Carpenter, 1982; Leem et al., 1993; Olausson et al., 2010). AM mainly consisted of Aδ-mechanoreceptors 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 (Treede et al., 1998; Djouhri and Lawson, 2004; Milenkovic
et al., 2008). Rapidly-adapting low-threshold \( \alpha \beta \)- and \( \alpha \delta \)-mechanoreceptors (D-hairs) were not studied. Receptive fields excited by von Frey hairs of more than 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 min later vehicle (SIF) or drug was applied. Stock solutions of fentanyl citrate (Sigma-Aldrich) 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 of 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 about 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 S.D. around the mean (AM: 61–150 %; CM: 73–131 %). We compared the sum of the spikes discharged to 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
Units were categorized as fentanyl-sensitive if their response decreased by more than 2 S.D. below the mean (i.e. < 61% for AM or < 73% for CM, respectively). Using this criterion, drug-induced effects were distinguished from normal variability due to repeated stimulation, similar to a previous study (Wenk et al., 2006). After > 10 min washout period, fentanyl-sensitive units were tested for naloxone 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 tested on 9 nociceptors from inflamed paws.

**Data acquisition and statistical analysis**

The signal driving the movement of the linear motor and raw electrophysiological data were collected with a Powerlab 6.0 system (AD Instruments, Spechbach, Germany), and spikes were discriminated off-line with the spike histogram extension of the software. The discharges of nociceptors to graded mechanical stimulation were compared between inflamed and normal tissue using two-way (for condition and stimulus intensity) repeated-measures (RM) ANOVA. If ANOVA revealed a significant effect, Bonferroni’s post-hoc test was used to compare groups.

The magnitude of the drug effect on the stimulus-response function of each mechanosensitive unit was quantified by calculating the area under the curve (AUC). For each unit, the baseline AUC values to stim 1 were compared to the AUC values obtained from stim 2 (vehicle or drug, respectively) (Fig. 1). The AUC transformed multivariate data (repeated measurements) into a univariate parameter to summarize the data and reduce the number of statistical
comparisons between and within groups. Mean values were compared by student paired t test for normally distributed data, or by Mann-Whitney or Kruskal-Wallis (followed by Dunn’s) tests for non-normally distributed data. The dose-response curves were fitted using Graphpad Software. The $\chi^2$-test was used for comparison of population proportions. For all tests a $P < 0.05$ was considered significant.
3. 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 ($P > 0.05$, 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 was increased in inflamed paws. We found 28% (26/91) of CM and 14% (8/56) of AM with an unprovoked ongoing activity in inflamed, versus 8% (6/80) CM and 0% (0/58) 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 to normal paws (0.54 ± 0.37 spikes/s) ($P = 0.038$, Mann-Whitney $U$ test). The AM discharge frequency in inflamed paws was 0.94 ± 0.32 spikes/s. We also found 6 (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. 2A and B). In 5 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 that 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 to 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 to noninflamed paws (CM: $89 \pm 9$ vs. $62 \pm 7$; spikes/stimulus ± SEM, 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: $148 \pm 29$ vs. $95 \pm 11$; spikes/stimulus ± SEM, Bonferroni post-hoc tests, $P < 0.05$, Fig. 3D).

$\alpha\beta$ nociceptors (4/25 noninflamed, 5/32 inflamed; CV $> 13$ m/s) were included in the analysis of AM since their stimulus-response plots did not significantly differ from $\alpha\delta$-fibers in both inflamed and normal paws. The response latencies were not different between inflamed and control paws (AM: $57 \pm 6$ ms vs. $53 \pm 7$ ms; CM: $93 \pm 11$ ms vs. $75 \pm 7$ ms; means ± SEM; $P > 0.05$, 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 C- and A-fiber nociceptors but not in LTM.

Fentanyl does not modulate discharges of nociceptor population innervating 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 $AUC_{\text{vehicle}}$ ratio ($AUC_{\text{vehicle}}/AUC_{\text{baseline}}$) and $AUC_{\text{fentanyl}}$ ratio ($AUC_{\text{fentanyl}}/AUC_{\text{baseline}}$), respectively. In control tissue, the mean $AUC_{\text{vehicle}}$ ratio was $1.03 \pm 0.14$ for CM (mean ±
S.D.; \( n = 8 \)); and 0.96 ± 0.11 for AM (mean ± S.D.; \( n = 7 \)). 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 1/14 CM and 1/12 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 4A-B, filled circle below horizontal dashed line). 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 to vehicle \((P > 0.05, \text{ Mann Whitney } U \text{ test, Fig 4C-D})\).

**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 \( \mu \)M and 25 \( \mu \)M. According to our definition of fentanyl sensitivity (see Methods), fentanyl inhibited 0/10 CM at 50 nM, 6/19 CM at 1 \( \mu \)M, and 10/28 CM at 25 \( \mu \)M concentration (Fig. 5A). In inflamed tissue fentanyl inhibited a significantly higher proportion of CM (6/19 at 1 \( \mu \)M; 10/28 at 25 \( \mu \)M) compared to normal tissue (1/14 at 25 \( \mu \)M)\((P < 0.05, \chi^2\text{-test})\). Similarly, fentanyl inhibited significantly more AM (2/6 at 1 \( \mu \)M; 7/17 at 25 \( \mu \)M) (Fig. 5B) in inflamed than in normal tissue (1/12 at 25 \( \mu \)M) \((P < 0.05, \chi^2\text{-test})\). Fentanyl evoked excitation of 1/10 CM at 50 nM, 3/19 CM at 1 \( \mu \)M and 5/28 CM at 25 \( \mu \)M and in 2/17 AM at 25\( \mu \)M concentration. Vehicle excited 1/10 CM and 1/8 AM compared to baseline.
To quantify the effects of fentanyl on the whole population of AM and CM nociceptors in inflamed skin, the mean AUC ratios (AUC_{fentanyl}/AUC_{baseline}) for each group and condition was compared to control (vehicle) (Fig. 5A-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 to 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. 5C-D). CM responses were inhibited by 2.9% at 50 nM up to 20% at 25 µM fentanyl. Compared to 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 IC_{50} value of 565 nM for CM and 930 nM for AM (Fig. 5E-F).

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 Kruskal-Wallis test followed by Dunn’s posthoc test, Fig. 6A-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 400µm 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 significantly decreased compared to 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).
4. Discussion

Our study demonstrates that the opioid receptor agonist fentanyl modulates the mechanical coding properties of both C- and A-fiber nociceptors and that this modulation becomes more prominent in chronic inflammation of the rat paw. This extends previous investigations in models of acute inflammation (Andrew and Greenspan, 1999). Wenk and colleagues showed that morphine could inhibit discharges in a substantial proportion (>50%) of C-fiber nociceptors when applied to their peripheral terminals 18 h after initiation of inflammation in vitro (Wenk et al., 2006). In agreement with the persisting mechanical hyperalgesia in our model of chronic inflammation (4 days) (Stein et al., 1988a), we observed significant mechanical sensitization in single A- and C-fiber nociceptors in vitro. By using a similar criterion for selecting opioid-responsive nociceptors as proposed by Wenk (Wenk et al., 2006), we now found that fentanyl suppressed the increased responses to maximal suprathreshold stimulation not only in C-, but also in 40% of A-fiber nociceptors. These data provide electrophysiological evidence that nociceptor sensitization and opioid sensitivity are not only detectable in acute injury but are maintained in persistent tissue inflammation.

Neurophysiological correlates of mechanical hyperalgesia

Subcutaneous FCA-induced inflammation results in mechanical hyperalgesia that starts as early as 4 h, reaches a maximum during the first 3 days and can last up to 14 days (Newbould, 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 are needed (Andrew and Greenspan, 1999; Du et al., 2003; Wenk et al., 2006). Although lower dosages of FCA can induce thermal without mechanical hyperalgesia (Iadarola et al., 1988; Fraser et al., 2000), the latter is considered more relevant in clinical conditions (Mantyh, 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 convincingly demonstrate 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 employing short-lived inflammatory stimuli no changes in mechanical thresholds could be detected (e.g. capsaicin, mustard oil, carrageenan) (Handwerker et al., 1987; Reeh et al., 1987; Baumann et al., 1991; Milenkovic et al., 2008). Skin incision or inflammatory models yielded controversial findings concerning activation thresholds (Andrew and Greenspan, 1999; Hämäläinen et al., 2002; Pogatzki et al., 2002; Wenk et al., 2006; Banik and Brennan, 2008). 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). Using electrical search techniques Wenk et al. 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 (>6 bars) (Meyer et al., 1991). The proportion of mechanically insensitive afferents (MIA) is about 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; Schmidt et al., 1995, 2000; Schmelz et al., 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 compared to (Handwerker et al., 1991; Treede et al., 1998).

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 (Handwerker et al., 1991; Treede et al., 1998). 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 mm$^2$ and 1 mm$^2$ in A-fiber nociceptors, but only with a 0.1 mm$^2$ probe in C-fibers (Andrew and Greenspan, 1999). In contrast, Wenk et al. applied mechanical stimuli of 3.3 (34 g/mm$^2$) and 5.5 bars (56 g/mm$^2$) with a probe of 4.9 mm$^2$ and did not observe significant differences in discharge rates between nociceptors from control and inflamed rat tissue (Wenk et al., 2006). We used a probe of 0.5 mm$^2$ 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 (Maekawa et al., 1994; Búzás and Cox, 1997; Coggeshall et al., 1997; Wang and Wessendorf, 2001; Busch-Dienstfertig and Stein, 2010). 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 are peptidergic and about 50% express 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 behavioural 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 there is an increase in neurons expressing opioid receptors, in receptors per neuron and a time-dependent upregulation of opioid receptor mRNA in the DRG with surges of μ-opioid receptor expression at 2 and 96 h (Puehler et al., 2004, 2006; Mousa et al., 2007). 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 (Yuge et al., 1985; Senami et al., 1986; Jaffe and Rowe, 1996; Picard et al., 1997; Grant et al., 2001). 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, while 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 have not been performed under inflammatory conditions. Thus, it is important to take into account all 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 was 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 1996) and humans (Wilder-smith 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 suppresses single nociceptor discharges to
intense mechanical stimuli. These data extend previous studies in that we now showed that a
substantial proportion of sensitized A-fiber nociceptors, in addition to C-fibers, are inhibited
by opioid agonists. These data provide electrophysiological evidence that nociceptor
sensitization and opioid sensitivity are not only detectable in acute injury but are maintained
in persistent tissue inflammation. This provides a basis for therapeutic opioid modulation of
chronic mechanical hyperalgesia in inflammatory conditions such as arthritis, fibromyalgia,
postoperative or cancer pain. Future studies will have to investigate what particular conditions might optimise and maximally augment this opioid receptor modulation of nociceptor sensitization in chronic inflammation.
References


Li JL, Ding YQ, Li YQ, Li JS, Nomura S, Kaneko T, Mizuno N. Immunocytochemical localization of mu-opioid receptor in primary afferent neurons containing substance P or...


**Figures**

FIG. 1. Scheme of the experimental protocol. A: An example trace of an 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 first stimulation series and 3 min later a second series of stimulation commenced. B: The responses of each single fiber were plotted and the AUC of the first (stim 1) and second (stim 2) stimulation series were calculated. C: The AUC ratio quantified fentanyl-induced changes in each unit.

FIG. 2. Patterns of discharges in nociceptors of FCA-inflamed paws. A: Electrical stimulation identified 5 units (numbered arrows) with one exhibiting irregular discharges. B: Spontaneous discharges in doublets (black points). C: Bursting discharges after gentle mechanical probing, example of a burst expanded. Time scale of bars: 5 s.

FIG. 3. FCA-induced inflammation increased the discharge rates of CM (A, B) and AM (C, D). Representative responses from a CM (A) and an AM (C) to 400 µm tissue indentation recorded from normal and inflamed paws. B: In CM the highest stimulus (400 µm) evoked significantly more responses in inflamed compared to normal paws (** P < 0.01, repeated-measures ANOVA followed by Bonferroni post-hoc test). D: AM from inflamed tissue fired significantly more responses to 200 and 400 µm displacements compared to normal tissue (* P < 0.05, *** P < 0.001, repeated-measures ANOVA followed by Bonferroni post-hoc test). Bars indicate S.E.M.

FIG. 4. Effects of fentanyl on CM and AM in normal paws (control). A and B: Scatter plots show the distribution of AUC ratios with the mean (horizontal line) for vehicle (open circles)
and 25 µM fentanyl (filled circles). The dashed horizontal line represents the cutoff below which units are considered fentanyl-sensitive. C and D: bar graphs demonstrate no significant inhibition by fentanyl (25 µM; CM, n = 14; AM, n = 12) on the entire nociceptor population (Mann-Whitney U test; ns: not statistically significant).

FIG 5. Effects of fentanyl on CM and AM in inflamed paws. A and B: Scatter plots show the distribution of AUC ratios with the mean (horizontal line) treated with vehicle (open squares), 25µM naloxone (open triangles), and 50 nM (grey squares), 1 µM (dark grey squares), and 25 µM (filled squares) fentanyl. Below the dashed horizontal line (lower cutoff limit) units were considered fentanyl-sensitive and tested with naloxone after a washout period. The average AUC ratio was significantly decreased by fentanyl 25 µM in both CM and AM (Mann-Whitney U test). C and D: Magnitude of inhibition by fentanyl on the whole CM and AM population. E and F: dose-response curves of fentanyl inhibition on CM and AM nociceptors. IC$_{50}$ = 565 nM for CM and IC$_{50}$ = 930 nM for AM. ns: not statistically significant. * P < 0.05.

FIG. 6. Naloxone prevents fentanyl-induced inhibition in inflamed paws. Bar graphs show AUC (means ± S.D.) 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 posthoc test; CM, n = 16; AM, n = 9). Nlx, Naloxone. * 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. B: No
significant changes in latencies of fentanyl-sensitive CM and AM fibers under fentanyl
without or with naloxone (Kruskal-Wallis test with Dunn’s posthoc test). C: Latencies of
fentanyl-insensitive AM but not of CM or LTM were significantly altered after addition of
fentanyl in inflamed tissue (Mann-Whitney U test, $P < 0.05$). Nlx: Naloxone.
Table 1: Physiological properties of mechanosensitive primary afferents from control and inflamed paws

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th></th>
<th>Inflamed</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>CV, ms⁻¹</td>
<td>vFT, mN</td>
<td>n</td>
<td>CV, ms⁻¹</td>
<td>vFT, mN</td>
</tr>
<tr>
<td><strong>A-Fibers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTM</td>
<td>10</td>
<td>20.9 ± 4.4</td>
<td>2.8</td>
<td>15</td>
<td>19.6 ± 4.3</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1.6/3.9)</td>
<td></td>
<td></td>
<td>(1.2/3.9)</td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>25</td>
<td>9.3 ± 4.9</td>
<td>19.6</td>
<td>54</td>
<td>7.4 ± 5.2</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(13.7/39.2)</td>
<td></td>
<td></td>
<td>(10.8/39.2)</td>
<td></td>
</tr>
<tr>
<td><strong>C-Fibers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>44</td>
<td>0.50 ± 0.12</td>
<td>19.6</td>
<td>99</td>
<td>0.58 ± 0.18</td>
<td>19.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(13.7-39.2)</td>
<td></td>
<td></td>
<td>(13.7-39.2)</td>
<td></td>
</tr>
</tbody>
</table>

CV is given in means and S.D. Median von Frey thresholds (vFT) with interquartile ranges are shown for each fiber category. No significant differences were found between the two conditions. LTM: low threshold mechanoreceptors; AM: A-fiber mechanonociceptors; CM: C-fiber mechanonociceptors.
FIG. 1

A

Mechanical stimulation series 1

Displacement, μm

10s 30s 40s 50μm 100μm 200μm 400μm

5 min

Mechanical stimulation series 2

Displacement, μm

10s 30s 40s 50μm 100μm 200μm 400μm

Fentanyl

B

Stimulus-Response plot series 1

Displacement, μm

Spike/stimulus

0 100 200 300 400

Stimulus-Response plot series 2

Displacement, μm

Spike/stimulus

0 100 200 300 400

C

AUC Fentanyl = 0.24

AUC μm x spikes

0 1 2 3 4 5

SIF

Stim1

Stim2
FIG. 2

A  Distance 28 mm

B

glass rod stimulation

C
FIG. 3

A

FCA

Control

Volts

Displacement, Frequency
Hz

μm

B

Displacement, μm

Spikes/stimulus

control (44)
FCA (54)

C

FCA

Control

Volts

Displacement, Frequency
Hz

μm

D

Displacement, μm

Spikes/stimulus

control (25)
FCA (32)
FIG. 4

A

AUC Ratio

CM

vehicle Fentanyl 25μM

B

AUC Ratio

AM

vehicle Fentanyl 25μM

C

Inhibition (%)

CM

vehicle Fentanyl 25 μM

D

Inhibition (%)

AM

vehicle Fentanyl 25 μM
FIG. 5

A) CM

B) AM

C) CM

D) AM

E) CM

F) AM
FIG. 6

A

AUC, μm x spikes

after wash

baseline 1
Fentanyl
baseline 2
Nlx/Fentanyl

n = 16

**

B

AUC, μm x spikes

after wash

baseline 1
Fentanyl
baseline 2
Nlx/Fentanyl

n = 9

**

**
FIG. 7

A

start
m. lat.

FIG. 7

B

Latency, ms

Baseline 1
Baseline 2
Nlx/Fentanyl
Baseline 1
Baseline 2
Nlx/Fentanyl

CM

AM

(16)
(9)
(39)
(9)
(20)
(10)

Latency, ms

CM

AM

LTM

(39)
(9)
(20)
(10)

Latency, ms

Baseline
Fentanyl
Baseline
vehicle
Baseline
Fentanyl
Baseline
Fentanyl

(16)
(9)
(39)
(9)
(20)
(10)

Latency, ms

Baseline
Fentanyl
Baseline
vehicle
Baseline
Fentanyl
Baseline
Fentanyl

(16)
(9)
(39)
(9)
(20)
(10)