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Authors
Hendrich, Jan
Alvarez, Pedro
Joseph, Elizabeth K
et al.

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Electrophysiological Correlates of Hyperalgesic Priming *In Vitro* and *In Vivo*

Jan Hendrich[a,c], **Elizabeth K Joseph**[a,c], **Xiaojie Chen**[a,c], **Oliver Bogen**[a,c], and **Jon D Levine**[a,b,c]

[a]Department of Oral and Maxillofacial Surgery, University of California San Francisco

[b]Departments of Medicine, University of California San Francisco

[c]Division of Neuroscience, University of California San Francisco

Abstract

We have modeled the transition from acute to chronic pain in the rat. In this model (termed hyperalgesic priming) a chronic state develops after a prior inflammatory process or exposure to an inflammatory mediator, in which response to subsequent exposure to prostaglandin E_2 (PGE_2) is characterized by a protein kinase Cε-dependent marked prolongation of mechanical hyperalgesia. To assess the effect of priming on the function of the nociceptor, we have performed *in vitro* patch clamp and *in vivo* single fiber electrophysiology studies using tumor necrosis factor to induce priming. *In vitro*, the only change observed in nociceptors cultured from primed animals, was a marked *hyperpolarization* in resting membrane potential (RMP); prolonged sensitization, measured at 60 minutes, could not be tested *in vitro*. However, complimentary with behavioral findings, *in vivo* baseline mechanical nociceptive threshold was significantly *elevated* compared to controls. Thirty minutes after injection of PGE_2 into the peripheral receptive field, both primed and control nociceptors showed enhanced response to mechanical stimulation. However, sixty minutes after PGE_2 administration the response to mechanical stimulation was further increased in primed but not in control nociceptors. Thus, at the level of the primary afferent nociceptor, it is possible to demonstrate both altered function at baseline and prolonged PGE_2-induced sensitization. Intrathecal antisense to $K_v7.2$, which contributes to RMP in sensory neurons, reversibly prevented the expression of priming in both behavioral and single-fiber electrophysiology experiments, implicating these channels in the expression of hyperalgesic priming.

Keywords

nociceptor; pain; hyperalgesia; action potential; tumor necrosis factor-alpha; prostaglandin e2; resting membrane potential; KCNQ

Corresponding author: Jon Levine (jon.levine@ucsf.edu), **Present / Permanent address:** Room C555, Campus Box 0440, 521 Parnassus Avenue, University of California at San Francisco, CA, USA, 94143-0440. Tel.: +1 415 476 5108; fax: +1 415 476 6305..

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INTRODUCTION

To study mechanisms underlying the transition from acute to chronic pain we developed a model in the rat, termed hyperalgesic priming, induced by exposure of the peripheral terminal of the primary afferent nociceptor to inflammatory mediators [1; 36]. Exposure (via intradermal injection) to these inflammatory mediators, such as tumor necrosis factor alpha (TNF) [33] or interleukin 6 (IL-6) [14]) produces a long-lasting neuroplastic change in nociceptor function, well beyond the hyperalgesia induced by these agents, which is observed as enhanced and markedly prolonged mechanical hyperalgesia in response to a subsequent exposure to an inflammatory mediator (e.g., by prostaglandin E_2 (PGE_2) [1]). In subsequent studies we demonstrated that this prolongation of the hyperalgesia induced by the pronociceptive inflammatory cytokine PGE_2 was mediated by protein kinase-C epsilon (PKC) [34]; PKA mediates the shorter-duration (30 min) prostaglandin E_2 (PGE_2) hyperalgesia in the control animal, and a similar period of time in the primed animal [1]. In these behavioral studies, altered function in the primary afferent nociceptor was inferred by two techniques. The first, reversal (of the longer-duration enhancement of PGE_2 hyperalgesia) by spinal intrathecal administration of antisense to signaling molecules implicated in priming [22; 34], demonstrates that the sensory neuron is the fundamental unit in priming. Secondly, peripheral administration of an inhibitor of PKC at the site of nociceptive testing demonstrates the dependence of the expression of hyperalgesic priming upon this intracellular signaling pathway [1].

To more directly evaluate the physiological changes in the nociceptor produced in the primed state, we performed *in vitro* patch clamp, and *in vivo* single fiber electrophysiology studies of primary afferent nociceptor function, to determine the electrophysiological correlates of the primed state. In these experiments we establish that, in addition to prolonged nociceptor sensitization, there is a change in baseline resting membrane potential and mechanical nociceptive threshold. Furthermore, the expression of these neuroplastic changes could be prevented by the intrathecal injection of oligodeoxynucleotide antisense to mRNA for $K_v7.2$, a member of the K_v7 family of voltage-gated potassium channels important for setting resting membrane potential (RMP), findings that suggest a role for $K_v7.2$ in chronic pain.

MATERIALS AND METHODS

Animals

Experiments were performed on adult male Sprague–Dawley rats (200–250 g; Charles River, Hollister, CA, USA). Animals were housed three per cage, under a 12-h light/dark cycle, in a temperature and humidity controlled environment. Food and water were available *ad libitum*. All experimental protocols were approved by the UCSF Committee on Animal Research and conformed to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Concerted effort was made to minimize the number of animals used and their suffering.

Drugs

Rat recombinant tumor necrosis factor (rr-TNFα) was purchased from R&D Systems (Minneanapolis, MN), PGE_2 was purchased from Sigma-Aldrich (St Louis, MO). The PKCε agonist ψεRACK (peptide-sequence HDAPIGYD) was synthesized by Biomatik (ON, Canada). The selection of the drug doses used in this study was based on dose–response curves determined during our previous studies [1; 33; 34; 41].

Induction of priming

Rats were briefly anesthetized with 2.5% isoflurane to facilitate the injection of rrTNFα or PGE_2 into the belly of the gastrocnemius muscle. The injection site was previously shaved and scrubbed with alcohol. Immediately after injections the skin puncture site was marked with a fine-tip indelible ink pen, so that the mechanical nociceptive threshold of the underlying injection site in the muscle could be repeatedly tested. Stock solutions of rrTNFα (5 ng/μl dissolved in 0.9% NaCl containing 0.5% BSA and stored at −20°C) and PGE_2 (1 μg/μl dissolved in 100% ethanol and stored at −80°C) were diluted in 0.9% NaCl immediately before injection.

Behavioral and *in vivo* electrophysiological experiments were performed 3 days after the administration of TNF or saline; *in vitro* electrophysiology was performed 5 - 8 days after intra-muscular administration of the retrograde tracer 1,1′-dioctadecyl-3,3,3′,3′-tertamethyllindocarbocyanine perchlorate (DiI) (2% w/v), along with TNF or saline.

Nociceptive testing

Mechanical nociceptive threshold in the gastrocnemius muscle was quantified using a Chatillon digital force transducer (model DFI2; AmetekInc., Largo, FL), as described previously [2]. Rats were lightly restrained in a cylindrical acrylic holder that allowed access to the hind limb through openings on either side of the restrainer. A 7-mm diameter probe attached to the force transducer was applied to the gastrocnemius muscle to deliver an increasing compression force; this probe width allows for selective evaluation of muscle pain (vis-à-vis overlying skin pain) [2]. The nociceptive threshold was defined as the force, in milliNewtons, at which the rat withdrew its hind leg. Nociceptive withdrawal threshold was defined as the mean of 2 readings taken at a 5-minute inter-stimulus interval. Each hindlimb was treated as an independent measure.

Electrophysiology

In vitro electrophysiology—*In vitro* whole-cell patch-clamp electrophysiology was performed on cultured male rat dorsal root ganglion (DRG) neurons as has been described previously [19]. The dorsal root ganglion (DRG) neurons innervating the gastrocnemius muscle were identified by their uptake of DiI, which was injected at the same time as the priming stimulus (TNF , 100 ng).

DRGs were surgically removed from rats 5-8 days after intramusclular DiI/ TNF injection, as has been described previously [19] and cultured in Neurobasal-A medium supplemented with 50-ng/ml nerve growth factor, 100 U/ml penicillin/streptomycin and B-27 (Invitrogen). Cells were plated on cover slips coated with poly-DL-ornithine (0.1 mg/ml) and laminin (5 μg/ml; Invitrogen), and incubated at 37°C in 5% CO_2 for between 2 and 30 h before use in experiments.

Whole-cell patch-clamp electrophysiology was performed using an Axopatch 200A amplifier and pCLamp 8.2 software (Molecular Devices, Sunnyvale, CA). Only DRG neurons <30μm in diameter (representing the nociceptive population) were used for the experiments, and were subjected to current-clamp after 2-36 hr in culture. Cover slips were incubated with FITC-conjugated *Griffoniasimplicifolia* isolectin B4 (Invitrogen) for 10 min before recording. Individual DRG neurons were held in the whole-cell configuration at −60 mV following seal formation (seal resistance > 1GΩ). Whole-cell capacitance and series resistance were compensated (80%) using the amplifier circuitry. Cells with a series resistance >10 MΩ were not used for experimentation. Following the switch to current clamp, current steps of 50 pA were applied to find the minimum stimulus intensity required to elicit an action potential stimulation (rheobase).

The external solution contained (in mM): NaCl (140), KCl (3), $MgCl_2$ (1), $CaCl_2$ (1), glucose (10), HEPES (10), adjusted to pH 7.3 and 320 mOsm. The pipette solution contained: KCl (140), EGTA (1); NaCl (10); MgATP (2); HEPES (10), adjusted to pH 7.3 and 310 mOsm. Traces were normalized to cell capacitance, as determined from the amplifier circuitry. Data were analyzed and plotted using Origin 6.1 software (OriginLab, Northampton, MA).

In vivo single fiber electrophysiology—The *in vivo* single fiber electrophysiology technique for studying muscle afferents has been described in detail previously [10]. In brief, rats were anesthetized with sodium pentobarbital (initially 50 mg/kg, intraperitoneally, with additional doses given to maintain areflexia throughout the experiment), their trachea cannulated to maintain patency of their upper airway, and heart rate monitored. Anesthetized animals were positioned right side down and an incision made on the dorsal skin of the left leg, between the mid-thigh and calf. Then the *b. femoris* muscle was partially removed to expose the sciatic nerve and gastrocnemius muscle. The edges of the incised skin were fixed to a metal loop to provide a pool that was filled with warm mineral oil, which bathed the sciatic nerve and gastrocnemius muscle.

The sciatic nerve was cut proximal to the stimulation electrode, to prevent reflex stimulation of muscles in the hind limb through reflex arcs during electrical stimulation of sensory neurons. Fine fascicles of axons were then dissected from the distal stump, and placed on a recording electrode. Single units were first detected by mechanical stimulation of the gastrocnemius muscle with a small blunt-tipped glass bar, which were subsequently confirmed by electrical stimulation of the mechanical receptive field according to the amplitude and duration of its action potential. Bipolar stimulating electrodes were then placed and held on the center of the receptive field of the muscle afferent by a micromanipulator (Narishige model MM-3, Tokyo, Japan). Conduction velocity of each fiber was calculated by dividing the distance between the stimulating and recording electrodes by the latency of the electrically evoked action potential. All recorded muscle afferents had conduction velocities in the range of type III (conduction velocity 2.5–30 m/s) or type IV (conduction velocity <2.5 m/s) muscle sensory fibers [13]. Mechanical threshold, determined with calibrated von Frey hairs (VFH Ainsworth, London, UK), was defined as the lowest force that elicited at least 2 spikes within 1 second, in at least 50% of trials. Sustained (60 s) suprathreshold (10 g) mechanical stimulation was accomplished by use of a mechanical stimulator that consisted of a force-measuring transducer (Entran, Fairfield, NJ, USA) with a blunt plastic tip that was applied by a micromanipulator (BC-3 and BE-8, Narishige) on the center of the afferent's receptive field, for 60 seconds. Neural activity and timing of stimulus onset and termination were monitored and stored on a computer with a Micro 1401 interface (CED, Cambridge, UK) and analyzed off-line with Spike2 software (CED).

Antisense oligonucleotide (ODN) preparation and administration

The phosphothioate antisense ODN for the KCNQ2 gene, 5′-ACCAGCGGGGAAAAAAAG-3′ was directed against a unique region of the rat mRNA sequence. The corresponding NCBI Genbank accession number and ODN position within the cDNA sequence are NM_133322 and 162-179. The antisense ODN is expected to downregulate the expression of all 9 different isoforms of the KCNQ2 gene according to database-entry O88943 (UniProtKB/SwissProt database). The mismatch ODN sequence, 5′-ACGAGTGGCGAGTAAACG-3′ corresponds to the antisense sequence with 6 bases mismatched (denoted in bold). A nucleotide BLAST was performed to confirm that the sequences were not homologous to other sequences in the rat.

ODNs were reconstituted in nuclease-free 0.9% NaCl (10μg/μl) and stored at −20°C until use. Prior to each injection, rats were anaesthetized with 2.5% isoflurane. A dose of 10 μg (injection volume 20 μl) of KCNQ2 antisense or mismatch ODN was administered using a 3/10 cc insulin syringe with a 29-gauge ultra fine ½-inch fixed hypodermic needle (Becton-Dickenson) inserted intrathecally, on the midline between the fourth and fifth lumbar vertebrae, once daily for three consecutive days. Previously demonstrated the down-regulation of several different proteins using this protocol have included the TTX-resistant sodium channel, $Na_v1.8$ [28], PLC 3 [22], gp130, a receptor subunit for IL-6 [40] or the polyadenylation element binding protein Cpeb [6].

Semi-quantitative one-step multiplex reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from cultured dorsal root ganglia treated with 1 μM of the phosphothioate antisense / mismatch oligonucleotides for 60 h in the presence of oligofectamine™ (Invitrogen) was extracted using Trizol reagent (Invitrogen) with the PureLink™ RNA mini kit (Ambion) according to the manufactures instructions. The amount of RNA was quantified with a spectrophotometer (UV-160, Shimadzu) and cDNA preparation was carried out with 1 μg of total RNA/sample and the superscript III platinum one-step RT-PCR system (Invitrogen). The PCR-primers used for the amplification of the rat KCNQ2 mRNA according to NCBI database-entry NM_133322 were: F2 = 5′-GCTTGCGGTTCTTACAAATC-3′ and B2 = 5′-GAATGAGACACCAATGAGGG-3′ (Invitrogen). To compensate for variations in the quality or quantity of the samples a one-step multiplex RT-PCR with S18 rRNA as an endogenous standard was performed and the amplification product of the KCNQ2 gene (317bp) was normalized to the PCR product of the S18 rRNA (489bp). Pilot experiments were performed to optimize for: 1. Annealing temperature (58.3°C). 2. Number of PCR cycles (35-45). 3. Ratio of S18 rRNA primer to competimers™ (Ambion; 3:7). The PCR-products were separated on 2% agarose gels and visualized by ethidium bromide. Images of the gels were acquired with the ChemImager system and analyzed with AlphaEaseFC Software (Alpha Innotech).

Statistics

Group data are expressed as mean ± SE of *n* independent observations. Statistical comparisons were made using GraphPad Prism 5.0 statistical software (GraphPad Software, La Jolla, CA). The Student's *t*-test was used to compare one or two independent samples, whereas analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests was used for comparing three or more samples. Data were tested for normality using the D'Agostino and Pearson omnibus normality test; if data did not pass the normality test for Gaussian distribution, Welch correction for the Student's *t*-test was used. Nonparametric data were analyzed using Wilcoxon's signed rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Hyperalgesic priming

The injection of a single dose (0.1 - 100 ng) of rat recombinant TNFα (rrTNFα) into the gastrocnemius muscle of separate groups of rats produced a dose-dependent mechanical hyperalgesia (Fig. 1A). Such hyperalgesia was present 1 hr after injection, reaching its peak by 2 hrs (Fig. 1B). While the injection of 0.1 ng of rrTNFα produced a mechanical hyperalgesia that was no longer present at 24 hrs (−4.0 ± 0.8 % change in mechanical threshold, P>0.05, Fig. 1B), 100 ng induced a hyperalgesia that was still significant at 24 hr (−24.9 ± 1.5 % change in mechanical threshold, $P < 0.001$, Fig. 1B), only reaching baseline by 48 hrs (−6.5 ± 2.0 % change in mechanical threshold, $P > 0.05$, Fig. 1B). The injection of

a single dose (1 - 100 ng) of PGE_2 into the gastrocnemius muscle also induced a dose-dependent muscle mechanical hyperalgesia (Fig. 1C). However, the mechanical hyperalgesia induced by PGE_2 reached its peak by 30 min after injection and lasted less than 4 hrs, at the doses assayed (Fig. 1D).

Three days after the i.m. injection of PGE_2 in doses of 10 or 100 ng (5 μl), rats were re-injected with 100 ng of PGE_2. In either case, no difference in the amplitude or duration of the hyperalgesia induced by the re-injection of 100 ng (5 μl) of PGE_2 (Fig. 1E) was observed. In contrast, the injection of PGE_2 (100 ng / 5 μl) in rats previously injected with a single dose of rrTNFα, but not in naïve rats, produced a significant prolongation of the mechanical hyperalgesia induced by PGE_2 (i.e., > 4 hrs), regardless of the dose of rrTNFα previously injected (Fig. 1F).

In vitro electrophysiology

Whole-cell patch-clamp electrophysiological recordings were made from acutely dissociated DRG neurons that had innervated the gastrocnemius muscle in primed and control rats, in order to determine if there was an effect of priming on membrane properties of DRG neurons. IB4+ neurons were selected for study because it is this population of nociceptors that mediates hyperalgesic priming [19; 23]. IB4+ neurons comprise roughly 70% of neurons retrogradely labeled from the gastrocnemius muscle [19]. All electrophysiological characteristics were measured from the rheobase (current threshold) action potential, with the exception of the number of action potentials fired (which was measured at 2X rheobase), and the resting membrane potential (which was measured immediately after obtaining the whole-cell configuration and switching to current clamp), shown in Figure 2.

There was no significant change in action potential after-hyperpolarization between primed and control neurons (−66.47 ± 2.74 mV (n=23) and 59.83 ± 2.32 mV (n=15), P=NS) respectively. There was also no significant difference between primed and control neurons in either action potential peak (55.20 ± 3.26 mV in primed (n=15), 54.24 ± 2.46 mV in control (n=23), P=NS) or duration (6.67 ± 0.96 ms in primed (n=15), 7.63 ± 1.04 ms in control (n=23), P=NS). We found no significant difference between the number of action potentials fired in neurons (in response to an 800 ms pulse at 2X rheobase) from primed (1.73 ± 0.32, n=15) and control (1.48 ± 0.20, n=23) rats (P=NS). In both naïve and primed rats, a very small minority of neurons produced more than one action potential.

There was, however, a marked difference between the resting membrane potential measured in DRG neurons derived from primed and control neurons (Fig. 2A). Primed neurons had a markedly more hyperpolarized RMP (−66.20± 2.45 mV, n=15) than control neurons (−51.22 ± 1.73 mV, n=23, P<0.001), as shown in Figure 2B. However, this difference did not translate into a change of electrical excitability of the neurons, *in vitro*. The membrane potential threshold for action potential generation, as assessed by a ramp protocol, was not significantly different between primed (−26.10 ± 2.97 mV, n=15) and control (−22.05 ± 1.83 mV, n=23) neurons (P=NS), as shown in Figure 2C. Also, the current threshold (rheobase) was similar between primed (501.2 ± 53.09 pA, n=15) and control (403.7 ± 42.20 pA, n=23) neurons (P=NS).

Effect of $K_V7.2$ antisense

Having found a change in RMP to be the only significantly changed biophysical variable in primed DRG neurons *in vitro*, we investigated the role of a class of ion channels implicated in setting RMP. $K_V7.2$ channels are important determinants of DRG RMP, and have potential roles in nociceptor function [12].

Knockdown of $K_v7.2$ RNA was confirmed in DRG cultures treated with antisense ODNs (Figure 3A). Antisense / mismatch ODNs were then administered intrathecally to selectively attenuate $K_v7.2$ before the priming stimulus (100 ng TNFα) was administered (intra-muscularly). Nociceptive behavioral testing was carried out after six days of antisense treatment, and four days after the priming stimulus. Rats treated with antisense displayed the same acute nociceptive response to PGE_2, a reduction in mechanical threshold that lasts less than 4 h. Antisense to $K_v7.2$ (Fig. 3A) was sufficient to selectively abolish the prolongation of the hyperalgesic response (~4 h) to PGE_2 that is the hallmark of hyperalgesic priming, but without affecting the early-phase of PGE_2 hyperalgesia (~30 min). Four days after the last dose of antisense (day 10), prolonged (~4 h) hyperalgesia returned. In the mismatch condition, no such abolition of the prolonged hyperalgesic response was observed (Fig. 3B) when measured on day seven.

In vivo electrophysiology

Baseline characteristics of primed neurons—Nociceptor fibers primed with TNF displayed a small but significantly higher ($P<0.05$) mechanical threshold (1.19 ± 0.10 g, n=35) compared to naive fibers (0.90 ± 0.08 g, n=44) as shown in Figure 4A ($P<0.05$). In rats treated with antisense, mechanical threshold was not significantly different from nociceptors in naive rats (0.90 ± 0.25 g, n=10).

The conduction velocity of primed fibers was not significantly different (2.20 ± 0.13 m/s, n=52) compared to naive fibers (2.23 ±0.18 m/s, n=44) , as shown in Figure 4B. Again, antisense treatment resulted in no significant change of conduction velocity (2.62 ± 0.43 m/s, n=11) compared to naive rats.

Response to prostaglandin E_2—While it was not possible to patch clamp DRG neurons for the >60 min required to examine prolonged sensitization in primed neurons, it was possible to do this in *in vivo* single fiber electrophysiology experiments. PGE_2 (100 ng intradermal injection adjacent to each fiber's mechanical receptive field) was administered in order to compare prolonged sensitization in primed and control neurons. The mechanical threshold and response to a prolonged (60 s) suprathreshold (10 g) mechanical stimulus were assessed before PGE_2, as well as 30 and 60 min after PGE_2 injection.

The time-course of mechanical threshold over 60 min following PGE_2 injection is shown in Figure 4C. While both the control (n=10) and primed (n=21) groups showed a decreasing mechanical threshold over 30 (1.18 ± 0.18 g and 1.03 ± 0.14 g respectively) and 60 (0.98 ± 0.15 g and 0.98 ± 0.11 g respectively) min, neither reached statistical significance compared to the threshold before PGE_2 injection (1.19 ± 0.18 g and 1.19 ± 0.14 g respectively). Antisense treatment (n=10) also caused no significant change in mechanical threshold with values of 1.15 ± 0.25 g at 30 min and 1.37 ± 0.39 g at 60 min compared to 0.90 ± 0.25 g before PGE_2 injection. The number of action potentials elicited by supra-threshold mechanical stimulation was also measured in primed rats. Sixty minutes after the injection of PGE_2 the number of spikes generated in response to the prolonged 10 g stimulus was significantly increased in C-fibers from both primed and control rats. However, the increase in firing was significantly larger in primed fibers (658.6 ± 169.5 spikes after 60 min compared to 230.8 ± 92.5 spikes before PGE_2 application, $P<0.01$) than in control fibers (236.2 ± 91.2 spikes after 60 min compared to 87.0 ± 26.7 spikes before PGE_2 application, NS), as shown in Figure 4D. The administration of $K_v7.2$ antisense (75.67 ± 25.43 spikes after 60 min) prevented the increase in nociceptor firing in response to PGE_2 observed in the primed condition. Response histograms of primed neurons to PGE_2 are shown in Figure 4E.

DISCUSSION

Hyperalgesic priming is a model of the transition from acute to chronic pain, in which a prior inflammatory insult enhances and markedly prolongs mechanical hyperalgesia in response to subsequent exposure to inflammatory mediators. We evaluated the changes in membrane properties associated with the development of the primed state, as a means of understanding this transition. While we have suggested that the transition from acute to chronic pain is associated with neuroplastic changes in the primary afferent nociceptor [5], the underlying changes in nociceptor function have been previously inferred from behavioral studies using pharmacological reagents administered at the sensory neruon receptive field [22; 34], or from intrathecal administration of oligodeoxynucleotide antisense to mRNA for second messengers involved in transition to the primed state [1].

A hallmark of hyperalgesic priming is that the two-phase behavioral response to PGE_2 is altered after PGE_2 application [1]. PGE_2 sensitizes nociceptors, reducing mechanical threshold over 30 min, with threshold returning to baseline after 60 min. In primed animals this sensitization lasts beyond four hours [36]. Using *in vivo* single-fiber electrophysiology, we confirmed that at 60 min after administration of PGE_2, primed nociceptors had a marked increase in their response to the suprathreshold mechanical stimulus when compared to the effect of PGE_2 on control nociceptors at the same time, correlating with our behavioral data.

In a previous study of the effect of inflammation on nociceptor function Wang and colleagues examined the mechanism by which PGE_2 induced sensitization of P2X3 in DRG neurons from rats exposed to complete Freund's adjuvant to induce inflammation 3-5 days previously [42]. While this sensitization was PKC as well as PKA dependent, compatible with the presence of hyperalgesic priming [1], we previously reported that for the tetrodotoxin-resistant sodium current contribution to nociceptor sensitization produced by PGE_2, *in vitro* (in the absence of prior inflammation), sensitization is already PKC as well as PKA dependent [18]. Therefore, in the present experiments we chose to first describe the baseline characteristics of nociceptor function induced by prior exposure to inflammation (i.e. the primed state), the behavioral implications of which were manifested in the response properties of the primed nociceptor to PGE_2.

In our characterization of membrane properties in nociceptors *in vitro*, the only electrophysiological alteration we observed was a marked, approximately 15 mV hyperpolarization of the resting membrane potential in the primed nociceptor. Although one might have expected that a hyperpolarization of resting membrane potential would increase action potential threshold, we observed no change in threshold for activation by a depolarizing current ramp. Electrical stimulation of cultured neurons is not, however, the optimal test for response to mechanical stimulation of nociceptors. Therefore, we also measured the response of primed nociceptive fibers to mechanical stimuli, *in vivo*. Here we found that primed nociceptors did in fact have a significantly higher mechanical threshold compared to unprimed control fibers. However, despite this higher mechanical threshold of primed nociceptors, treatment with PGE_2 caused a much greater increase in nociceptive firing response to 10 g stimulation in primed fibers compared to that observed in naive nociceptors.

The current study raises questions about the long-term changes that arise in nociceptors, which manifest as a hyperpolarization of RMP (without other changes in biophysical properties in the same cell) and contribute to the higher basal mechanical threshold, but enhanced response to mechanical stimulation in nociceptors after exposure to PGE_2. Our *in vitro* data, revealing a hyperpolarization of resting membrane potential in nociceptors from primed rats, support the idea that neuroplastic changes underlie the phenotype of the primed

nociceptor. One family of voltage-gated potassium channels, K_V7, is well-established to be important for the maintenance of RMP in neurons [7] and also has a role in determining the excitability of DRG neurons [35]. K_V7 channels produce a current (called 'M current') that activates around the resting membrane potential of DRG neurons, and serves to limit the excitability of neurons by hyperpolarizing them.

Therefore we evaluated the role of $K_v7.2$ in order to determine whether this channel was involved in the expression of the primed state. Administration of antisense to $K_v7.2$ prevented the prolongation of PGE_2 hyperalgesia in the primed rat, without affecting the early phase of PGE_2 hyperalgesia, an effect that reversed after cessation of antisense administration. Similarly in single fiber electrophysiology studies we found that oligodeoxynucleotide antisense to $K_v7.2$ mRNA reversed the enhanced response of C-fiber nociceptors 1 h after injection of PGE_2 into their peripheral receptive field. These findings provide the first evidence for a role of a specific ion channel family (K_v7) in the prolonged PGE_2 hyperalgesia in the primed rat, which differ significantly from those involved (via PKA activation) in the early phase of PGE_2 hyperalgesia [9; 30; 31; 42; 43].

While these data demonstrate a dependence of the prolonged hyperalgesia on $K_v7.2$, they do not prove that the late-phase hyperalgesia is actually mediated by ion flux through $K_v7.2$ channels themselves. K_v7 channels have been shown to dampen neuronal excitability [8; 32]. The suppression of K_V7 channels has been shown to lead to increased neuronal excitability and sensitivity to pain [32; 38], whereas the use of the K_V7 channel opener retigabine can cause nociceptor hyperpolarization and attenuate nociceptive behaviors [4; 29]. Indeed, the establishment of a model of bone cancer pain relies on the hyperexcitability of sensory neurons caused by K_v7 channel inhibition [45]. Therefore it might seem counter-intuitive that administration of antisense to these channels would prevent the expression of a chronic pain state. One explanation might be that channels from other K_v7 subclasses may dynamically compensate for the down-regulated $K_v7.2$, thus preventing an increase in excitability. Examples of K_v7 channel upregulation as a compensation mechanism include oxidative stress conditions [16] and axotomy [39]. Other K_v7 channels, such as $K_v7.3$ (which forms heteromers with $K_v7.2$) and $K_v7.5$ which have been demonstrated to be present in DRG neurons [27; 32], also remain to be evaluated in their relevance to the expression of hyperalgesic priming.

These data also suggest that $K_v7.2$ mRNA may play a role other than that of the conventional biophysical role of increasing $K_v7.2$ channels at the cell membrane leading to dampened excitability. It is clear that hyperalgesic priming is the result of a complex network of intracellular processes with a central role of PKCε activation [1; 15; 25], and has only previously been disrupted by applying protein translation inhibitors [6]. Therefore it will be worthwhile to investigate the intracellular pathways involved in K_v7 modulation / expression to see if they ally with some of the intracellular pathways we have described for hyperalgesic priming [36].

While further work is required to fully determine the role of K_v7 (and possibly other classes of) channels responsible for the expression of neuroplastic changes in RMP and increased mechanical threshold in nociceptors, our lab has demonstrated an increase in TTX-R sodium current as a result of PKC activation [17; 26]. Also, activation of PKCε has been established to cause an increase in function (by phosphorylation) of the TTX-R sodium channel $Na_V1.8$, leading to mechanical hyperalgesia [44]. Indeed, up-regulation of Na_v channels (including $Na_v1.3$ and $Na_v1.8$) has been observed upon exposure to inflammatory mediators such as TNFα and carageenan [3; 11]. Therefore, the interplay of Na_v and K_v7 channels should be investigated.

We have recently shown that DRG neurons from female rats also have a more hyperpolarized RMP than those from male rats, along with a higher mechanical threshold for activation *in vivo* [20]. Of note, estrogen, which regulates sexual dimorphism in nociceptor function [21] has been shown to regulate K_V7 potassium channel expression in neuropeptide Y-ergic neurons [37]. Since hyperalgesic priming is restricted to male rats [24] and administration of estrogen abolished the establishment of priming in ovariectomized female rats, it would be of interest to assess the role of K_V7 channels in female rats as an ion channel end-point of estrogen control of nociceptor function that causes the phenotype seen *in vivo*.

In conclusion, we have shown that primed nociceptors have a hyperpolarized RMP *in vitro*, as well as an increased baseline mechanical threshold but an increased response to a pronociceptive inflammatory mediator *in vivo*. This work provides evidence for a long-lasting re-organization of ion channel function, including $K_v7.2$ which contributes to RMP, as a result of a brief inflammatory stimulus 72 hours previously.

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Summary

We have made an electrophysiological characterization of the transition from acute to chronic pain, termed hyperalgesic priming. Neuroplastic changes in the nociceptor arising from an acute inflammatory insult can lead to enhanced and prolonged subsequent responses to painful stimuli.

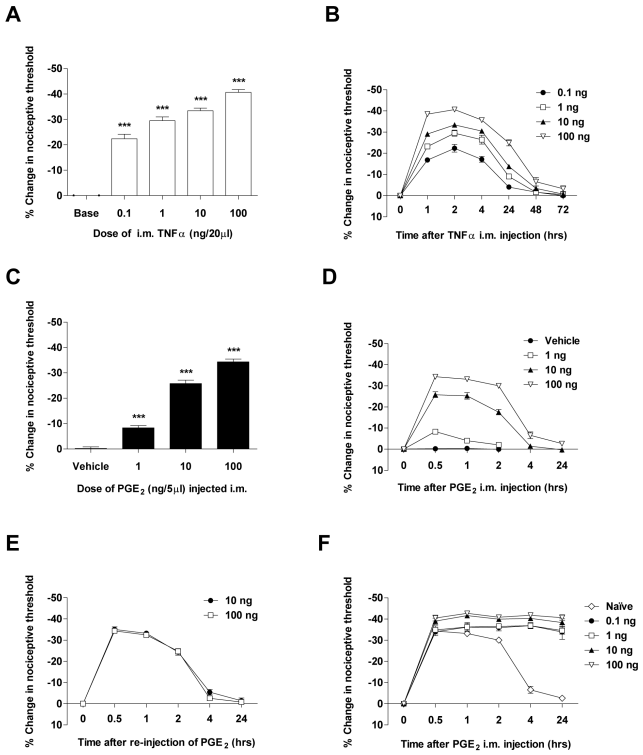

Figure 1.

Injection of TNFα into the gastrocnemius muscle produces mechanical hyperalgesia and hyperalgesic priming. (**A**) Single dose of rat recombinant TNFα (0.1-100 ng) injected into the gastrocnemius muscle of different groups of rats decreased the mechanical nociceptive threshold in a dose-dependent manner, in readings taken 2 hrs after its injection (n=6 per group). (**B**) The time-response curve for hyperalgesia induced by different doses of TNFα (n=6 per group). Muscle mechanical hyperalgesia reached a maximum 2 hrs post-injection (100 ng), and lasted up to 48 hrs. (**C**) Single doses of PGE_2 (1-100 ng) injected into the gastrocnemius muscle produced a dose-dependent decrease in mechanical nociceptive in readings taken 30 min after injection (n=6 per group). (**D**) Mechanical hyperalgesia induced by different doses of PGE_2 was investigated from 30 min to 24 hrs (n=6 per group). Hyperalgesia reached a maximum 30 min post-injection and lasted less than 4 hrs. (**E**) Previous injection of either 10 or 100 ng of PGE_2, three days prior, did not change the amplitude or duration of mechanical hyperalgesia induced by a re-injection of 100 ng (5 μl) of PGE_2 in the same injection point (n=6 per group). (**F**) Rats previously injected with TNFα exhibited prolonged duration of PGE_2 (100 ng)-induced mechanical hyperalgesia compared to naïve rats (n=6). ***$P < 0.001$.

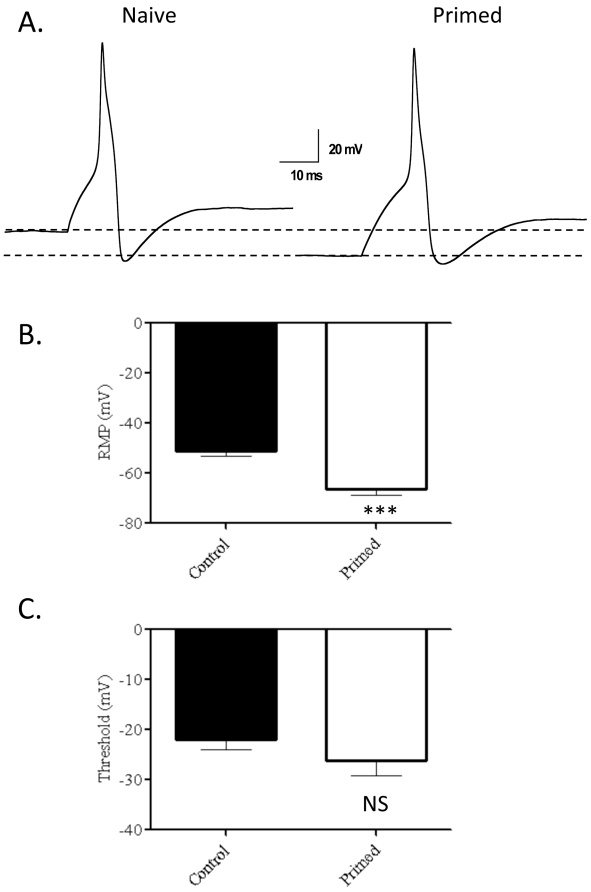

Figure 2.

Action potential characteristics from primed DRG neurons *in vitro*. Representative traces (**A**) from naive (n=23) and primed (n=15) neurons show a significant hyperpolarization of resting membrane potential (RMP) in primed neurons (**A, B**), which occurs in the absence of any significant difference in other characteristics, for example membrane threshold of action potential generation (**C**). ***P<0.001

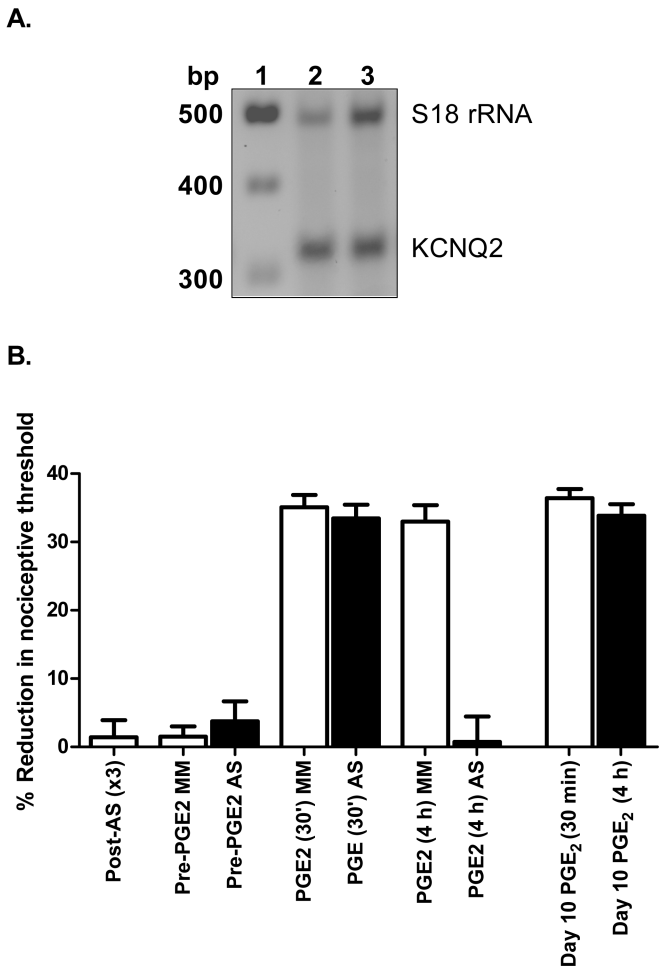

Figure 3.

(**A**). Knockdown of the KCNQ2 expression in cultured dorsal root ganglia. Cultured DRGs treated with 1μM antisense ODN for 60 h in the presence of oligofectamine™ demonstrate a 27.5 ±1% decrease over all amplification cycles in their mRNA expression compared to cultured DRGs treated with 1 μM mismatch ODN. Column 1- DNA ladder, column 2- mismatch ODN, column 3- antisense ODN. (**B**) Application of $K_V7.2$ antisense prevents the expression of priming. $K_V7.2$ antisense (n=10) or mismatch (n=6) injections (10 μg intrathecal) were given daily for three days. On day four, nociceptive testing was performed in the antisense condition. The TNFα priming stimulus was applied (100 ng) followed by three more days of antisense / mismatch. On day seven, testing was performed before, 30 min and 4 h after PGE_2 (100 ng) injection. Antisense treatment prevented the reduction in threshold observed 4 h after PGE_2 injection. Nociceptive testing was performed again on Day 10 in antisense-treated rats (three days after cessation of treatment), the prolonged hyperalgesia 4 h after PGE_2 injection had returned.

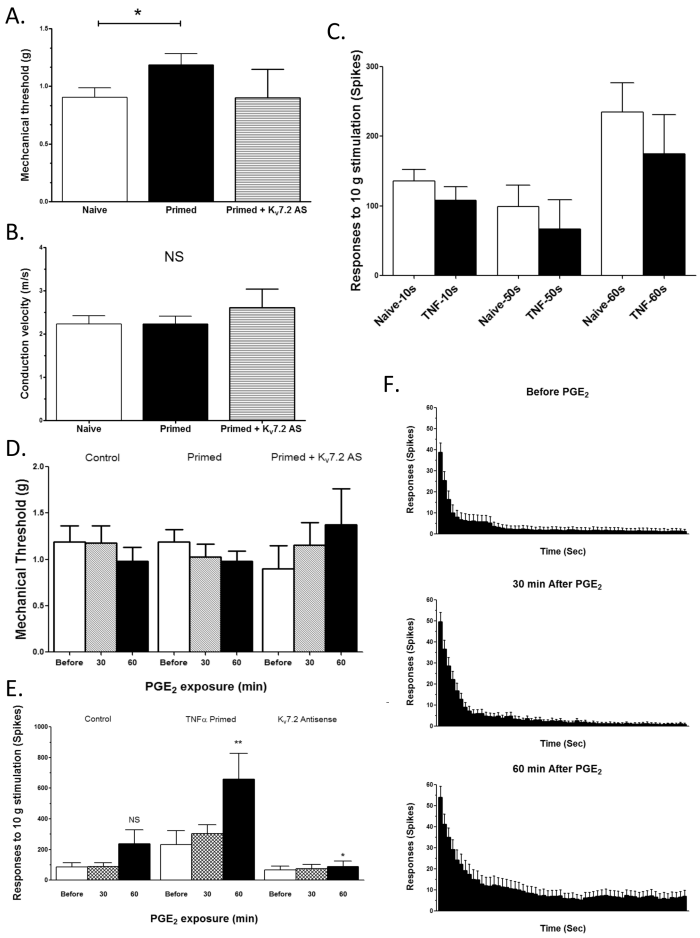

Figure 4.

A measurement of the excitability of naive and primed nociceptive fibers *in vivo*. (**A**) C-fibers in primed neurons (n=35) displayed a significantly elevated mechanical threshold compared to naive fibers (n=44), that was reversed by antisense (n=10), but a similar conduction velocity (**B**). (**C**) Upon exposure to 100 ng 35 PGE_2, mechanical threshold was not significantly altered in either primed (n=21) or $K_V7.2$ antisense-treated (n=10) fibers compared to naive fibers (n=10) after 60 min. (**D**) Compared to control fibers (n=10), the spike frequency at 60 min was increased in primed fibers (n=21) and decreased in primed fibers treated with antisense (n=10). (**E**) Spike response histogram of primed fibers measured before 100 ng PGE_2 application, as well as 30 and 60 min after. *P<0.05, **P<0.01