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Article **Neuroscience**

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Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype

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Blockade of prostaglandin (PG) production by COX inhibitors is the treatment of choice for inflammatory pain but is also prone to severe side effects. Identification of signaling elements downstream of COX inhibition, particularly of PG receptor subtypes responsible for pain sensitization (hyperalgesia), provides a strategy for better-tolerated analgesics. Here, we have identified PGE_2 receptors of the EP2 receptor subtype as key signaling elements in spinal inflammatory hyperalgesia. Mice deficient in EP2 receptors ($EP2^{-/-}$ mice) completely lack spinal PGE_2-evoked hyperalgesia. After a peripheral inflammatory stimulus, $EP2^{-/-}$ mice exhibit only short-lasting peripheral hyperalgesia but lack a second sustained hyperalgesic phase of spinal origin. Electrophysiological recordings identify diminished synaptic inhibition of excitatory dorsal horn neurons as the dominant source of EP2 receptor–dependent hyperalgesia. Our results thus demonstrate that inflammatory hyperalgesia can be treated by targeting of a single PG receptor subtype and provide a rational basis for new analgesic strategies going beyond COX inhibition.

Introduction

Classical COX inhibitors, also known as NSAIDs, are among the most frequently used analgesics (for a review see ref. 1). They inhibit PG synthesis through nonselective blockade of constitutively expressed COX-1 and inducible COX-2 and display, in addition to their analgesic effect, antiinflammatory and antipyretic properties. Unfortunately, in particular their long-term use is often hampered by severe side effects, including gastric ulcerations. It is generally accepted that both their desired and their unwanted (side) effects originate from the global block of PG production. More recently developed COX-2–selective inhibitors (or coxibs) proved analgesic and antiinflammatory both in experimental models (2) and in patients (e.g., ref. 3). However, recent evidence suggests that the prolonged use of these COX-2–selective inhibitors also confers significant risks to patients, as it may predispose to severe cardiovascular events, such as heart attack and stroke (4, 5). The identification of new therapeutic targets downstream of COX inhibition may therefore provide a rational and promising strategy for the development of more specific and better-tolerated analgesics.

Prostaglandin E_2 (PGE_2) is a key factor in the generation of exaggerated pain sensations evoked by inflammation (6). It exerts its cellular effects through 4 different G protein–coupled receptors encoded by separate genes, termed EP1 through EP4 (7). These receptors differ in their tissue distribution, signaling pathways, and physiological functions, which should allow the treatment of inflammatory pain with much greater specificity than currently achievable by the global blockade of PG synthesis via COX inhibitors. Studies performed either in mutant mice lacking individual PG receptors (8–10) or with synthetic PG receptor ligands (e.g., refs. 11, 12) have not yet provided a coherent picture of which EP receptors are responsible for inflammatory pain sensitization. This is partly due to the fact that PGs facilitate nociception at different levels of integration (13). They do not only sensitize peripheral nociceptors (14–16) but can also lead to changes in the central, particularly spinal, processing of nociceptive input (17, 18). It is hence still unclear which PGs and which PG receptors mediate pain sensitization in the periphery and in the spinal cord, respectively, and to what extent the 2 sites contribute to inflammatory hyperalgesia.

During recent years several cellular candidate pathways have been identified that are possibly involved in PG-induced pain sensitization in the periphery (14, 16) and in the CNS (19, 20). Our own group has suggested that PGE_2 facilitates spinal nociceptive transmission through blockade of inhibitory glycine receptors located in the superficial layers of the spinal cord dorsal horn (20, 21). This blockade would lead to a disinhibition of dorsal horn neurons and subsequently facilitate the propagation of nociceptive signals through the spinal cord to higher CNS areas. We now demonstrate that mice deficient in the EP2 receptor ($EP2^{-/-}$ mice) not only completely lack PGE_2-mediated inhibition of glycinergic neurotransmission but also show no pain sensitization after intrathecal PGE_2 injection. In contrast to spinal pain sensitization, peripheral pain sensitization evoked by subcutaneously injected PGE_2 was retained in $EP2^{-/-}$ mice. In the zymosan A model of peripheral inflammation, $EP2^{-/-}$ mice exhibited an almost normal early hyperalgesia. However, unlike WT and $EP3^{-/-}$ mice, $EP2^{-/-}$ mice completely recovered from sensitization within 2 days, indicating that spinal processes dominate peripheral ones during prolonged inflammatory pain sensitization.

Nonstandard abbreviations used: ACSF, artificial cerebrospinal fluid; BAC, bacterial artificial chromosome; EGFP, enhanced GFP; GlyT2, glycine transporter type 2; IPSC, inhibitory postsynaptic current.

Conflict of interest: The authors have declared that no conflict of interest exists.

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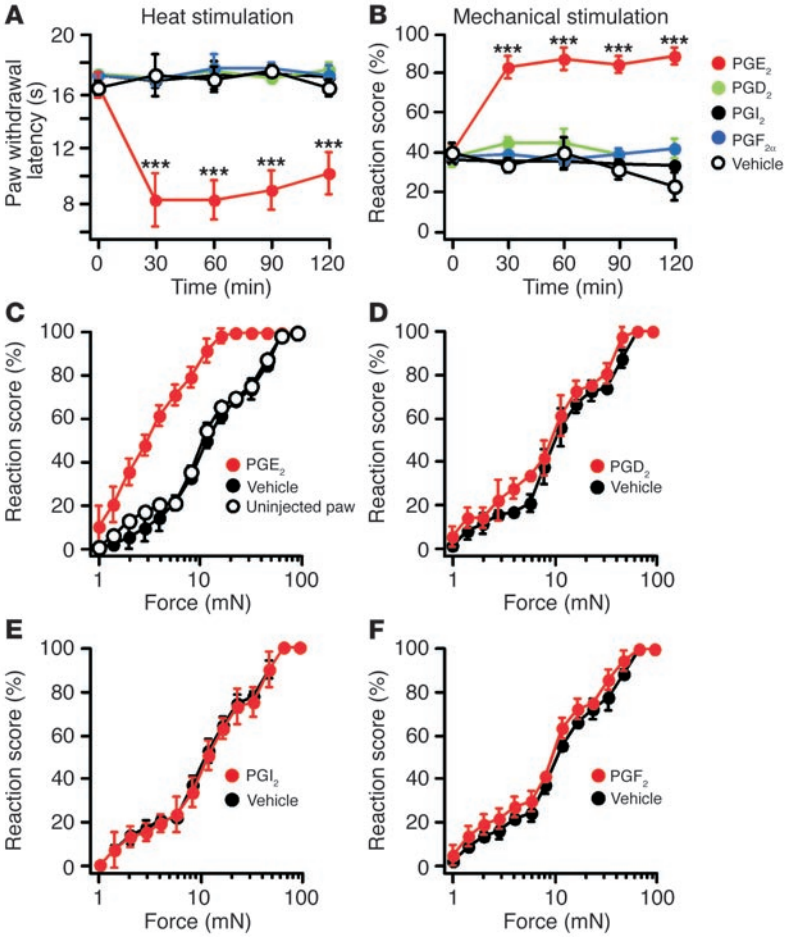

Figure 1
Thermal and mechanical sensitization by different spinally applied PGs. (**A**) Changes in paw withdrawal latency (mean ± SD, n = 4–6) upon exposure of mice to a defined radiant heat stimulus after intrathecal injection of PGE_2, PGD_2, PGI_2, $PGF_{2\alpha}$ (0.2 nmol per mouse), or vehicle (1% ethanol). (**B**) Changes in nociceptive reactions (percent maximum possible response, mean ± SD, n = 4–6; for details see Methods) upon mechanical stimulation with an 8-mN von Frey filament after intrathecal PG injection. The legend in **B** refers to **A** and **B**. (**C**–**F**) Stimulus-response curves for mechanical stimulation obtained before and 60 minutes after intrathecal injections of the different PGs (or vehicle) (same mice as in **B**). ***$P < 0.001$ (ANOVA followed by Scheffe's post hoc test, n = 6 each).

Results

Spinal hyperalgesic properties of different PGs. In a first series of experiments, we determined the ability of different PGs to induce spinal pain sensitization. We injected small amounts (0.2 nmol per mouse) of PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2, or vehicle (1% ethanol) intrathecally (i.e., into the spinal canal) in WT mice and monitored changes in their nociceptive reactions upon exposure to a defined noxious heat stimulus or to mechanical stimulation with von Frey filaments (Figure 1). Following intrathecal injection of PGE_2, pronounced thermal hyperalgesia developed within less than 30 minutes and recovered slowly over about 6 hours (see also Figure 2). At the peak of the response (60 minutes after injection), paw withdrawal latencies upon thermal stimulation decreased from 16.4 ± 0.9 seconds to 8.3 ± 1.4 seconds (mean ± SD, n = 6) (Figure 1A). All other PGs were without significant effect. Similar results were obtained for mechanical stimulation (Figure 1B). PGE_2 increased mechanical sensitivity significantly over the entire range of stimulation strengths tested (1–90 mN). PGD_2, $PGF_{2\alpha}$, and PGI_2 were without effect (Figure 1, C–F).

EP2 receptors mediate the spinal hyperalgesic effect of PGE_2. Among the 4 subtypes of EP receptors, expression in the spinal cord is best documented for the EP2 and EP3 subtypes (22–24). We therefore determined the contribution of these receptors to spinal PGE_2-induced hyperalgesia. Under base-line conditions, WT mice and EP2 receptor– and EP3 receptor–deficient mice ($EP2^{-/-}$ and $EP3^{-/-}$ mice) showed virtually identical sensitivities to noxious heat ($P > 0.5$, ANOVA followed by Scheffe's post hoc test, n = 6 each) and mechanical stimulation ($P > 0.19$) (Figure 2, A and B) and no abnormalities in the expression of markers of the spinal nociceptive system (Supplemental Figure 1; available online with this article; doi:10.1172/JCI200523618DS1). Following intrathecal injection of PGE_2, $EP3^{-/-}$ mice developed thermal and mechanical hyperalgesia indistinguishable from that in WT mice (Figure 2, A–C and E). In contrast, PGE_2 failed to induce thermal or mechanical sensitization in $EP2^{-/-}$ mice. The defect in mechanical sensitization occurred throughout the entire range of stimulation strengths tested (Figure 2D).

In contrast to spinal hyperalgesia, peripheral sensitization was retained in $EP2^{-/-}$ (and $EP3^{-/-}$) mice (Figure 3). Thermal sensitization in $EP2^{-/-}$ and $EP3^{-/-}$ mice evoked by local s.c. injection of 0.5 nmol PGE_2 into the left hind paw was indistinguishable from that seen in WT mice. Mechanical sensitization was reduced in $EP2^{-/-}$ mice by 48% ± 8.2% (n = 6), which suggests that mechanical pain sensitization was partially mediated by peripheral EP2 receptors. $EP3^{-/-}$ mice behaved normally in both tests.

Contribution of EP2 receptors to spinal hyperalgesia evoked by peripheral inflammation. The lack of spinal PGE_2-mediated pain sensitization in $EP2^{-/-}$ mice in the presence of retained peripheral sensitization allowed us to determine the relative contributions of spinal versus peripheral processes to inflammatory pain sensitization. We therefore analyzed the different types of mice in the zymosan A model (Figure 4). In these experiments the yeast extract zymosan A (0.06 mg in 20 μl PBS) was injected s.c. into the plantar side of the left hind paw, a procedure that induces inflammation and a subsequent increase in spinal COX-2 expression (25). WT mice and the 2 types of mutant mice exhibited virtually identical paw swelling ($P > 0.69$, ANOVA followed by Scheffe's post hoc test, at 6 hours after zymosan A injection) (Figure 4A) and spinal COX-2 induction determined by real-time RT-PCR (Figure 4B). However, thermal and mechanical hyperalgesia developed differently in the different types of mice. In WT mice, paw withdrawal latencies decreased from 17.0 ± 0.3 seconds to 8.5 ± 1.2 seconds (n = 6) within 4 hours, remained stable for about 24 hours, and then recovered slowly within 7 days. Initially (at 2 hours), thermal and mechanical sensitization in $EP2^{-/-}$ mice was very similar to that seen in WT mice. However, from 4 hours onward $EP2^{-/-}$ mice recovered much faster from hyperalgesia, and a significant difference between WT mice and $EP2^{-/-}$ mice became obvious at 4–6 hours. From day 3–4 onward, thermal hyper-

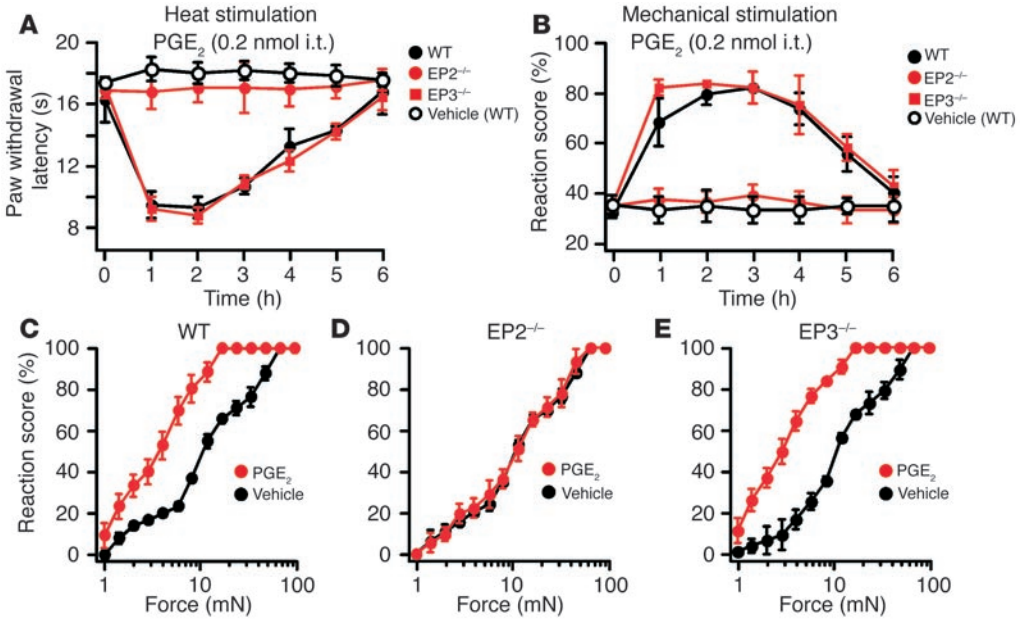

Figure 2
Thermal and mechanical hyperalgesia evoked by PGE_2 in $EP2^{-/-}$ and $EP3^{-/-}$ mice. (**A** and **B**) Paw withdrawal latencies (mean ± SD, $n = 6$ each) upon radiant heat stimulation (**A**) and nociceptive reaction scores (mean ± SD, $n = 6$ each) upon mechanical stimulation with an 8-mN von Frey filament (**B**) after intrathecal (i.t.) injection of PGE_2 (0.2 nmol). Nociceptive sensitization in $EP2^{-/-}$ mice was significantly less than in WT mice at all time points between 1 and 5 hours ($P < 0.001$, ANOVA followed by Scheffe's post hoc test, $n = 6$ each). (**C**–**E**) Stimulus-response curves for mechanical stimulation obtained before and after intrathecal injections of PGE_2 in WT mice and $EP2^{-/-}$ and $EP3^{-/-}$ mice (same mice as in **B**).

algesia in $EP2^{-/-}$ mice became statistically indistinguishable ($P > 0.05$, ANOVA) from that in vehicle-injected control mice. Similar effects were obtained for mechanical hyperalgesia (Figure 4, E and F).

We next tested whether the fast recovery from hyperalgesia seen in $EP2^{-/-}$ mice was due to the defect in spinal sensitization. Indeed, the time point at which sensitization in $EP2^{-/-}$ mice started to significantly differ from that in WT mice correlated well with the induction of COX-2 mRNA and PGE_2 concentrations in the spinal cord dorsal horn (Figures 4B and 5B, respectively). To verify the spinal origin of the delayed sensitization, we tested the effect of COX-2 inhibition in WT mice at different time points after zymosan A injection (Figure 5). Intrathecal injection of the COX-2–specific inhibitor celecoxib (2 or 20 nmol) caused only a modest antinociception at 2 hours after zymosan A injection, while at 6 hours and after 2 days a significant, dose-dependent and reversible antinociception was obtained. Interestingly, the reduction in thermal hyperalgesia achieved with intrathecal celecoxib was very similar to that resulting from the disruption of the EP2 receptor gene (compare Figure 4, C and D, and Figure 5).

EP2 receptor activation disinhibits superficial dorsal horn neurons. In a final set of experiments, we addressed the molecular mechanisms responsible for EP2 receptor–dependent spinal hyperalgesia. It was indeed the EP2 receptor subtype that was responsible for the inhibition of glycinergic neurotransmission by PGE_2 (Figure 6), a phenomenon that we have shown underlies spinal inflammatory hyperalgesia (21). Whole-cell patch-clamp recordings of inhibitory postsynaptic currents (IPSCs) mediated by glycine were made from neurons located in the superficial spinal cord dorsal horn, where most nociceptive afferent nerve fibers terminate. As shown previously, in WT mice PGE_2 (1 μM) reduced the amplitudes of glycinergic IPSCs by 39.9% ± 5.0% (mean ± SEM, $n = 8$). This inhibition was absent in $EP2^{-/-}$ mice (–8.5% ± 6.5%, $n = 9$) but remained largely unchanged in $EP3^{-/-}$ mice (29.7% ± 6.1%, $n = 8$), demonstrating that the reduction of glycinergic IPSCs by PGE_2 was exclusively mediated through EP2 receptors.

Because inhibition of glycinergic neurotransmission by PGE_2 occurs in the majority (about 80%) of dorsal horn neurons but not in all of them (20), we tested whether this inhibition was restricted to a defined subset of neurons (Figure 7). To address this question, we recorded glycinergic IPSCs in spinal cord slices obtained from bacterial artificial chromosome (BAC) transgenic mice, which express

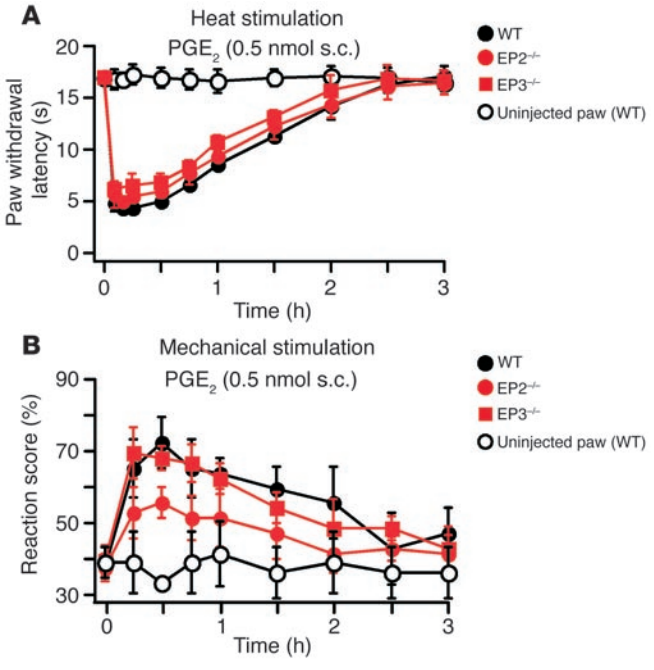

Figure 3
Peripheral pain sensitization. Thermal sensitization (paw withdrawal latencies upon exposure to noxious heat) (**A**) and mechanical sensitization (reaction scores evoked by stimulation with an 8-mN von Frey filament, mean ± SD, $n = 6$ each) (**B**) at different time points after s.c. injection of PGE_2. $EP2^{-/-}$ mice exhibited significantly less mechanical sensitization than WT mice at time points from 0.5–1.5 hours ($P < 0.01$–0.05, ANOVA followed by Scheffe's post hoc test).

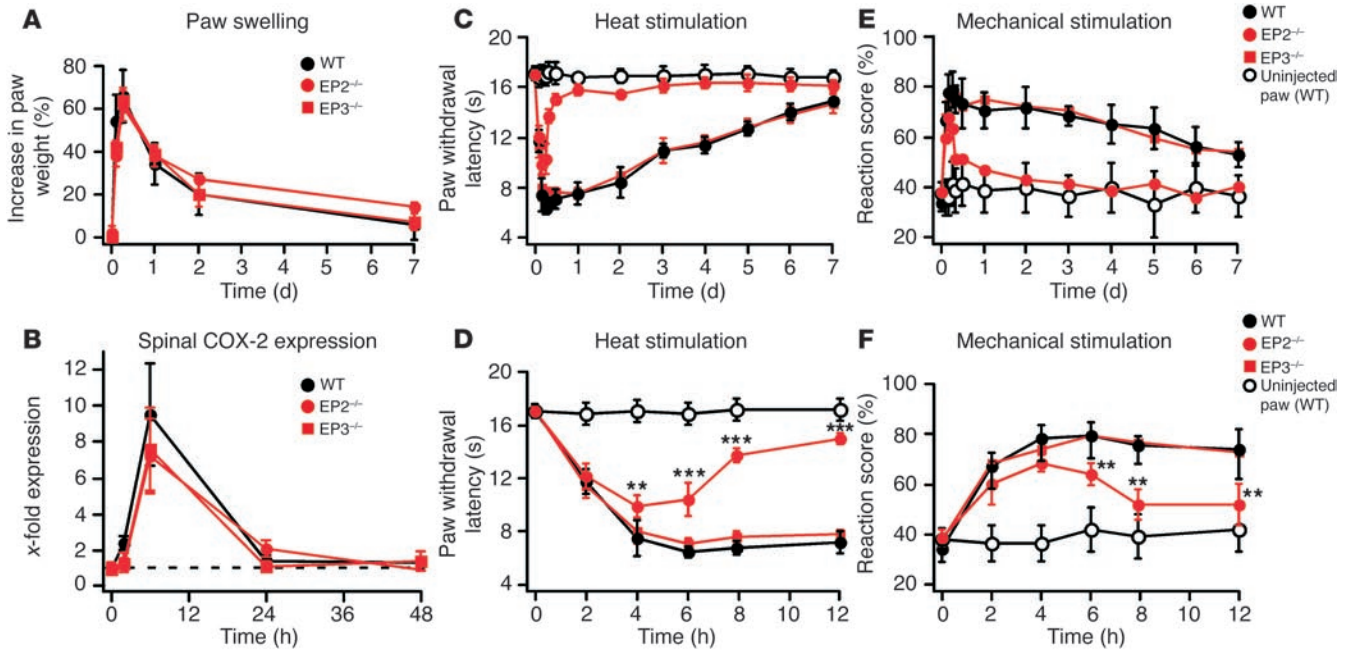

Figure 4
Paw swelling, spinal COX-2 mRNA induction, and nociceptive sensitization after zymosan A injection. (**A**) Percent increase in paw weight (mean ± SD, $n = 6$ each) after s.c. injection of 0.06 mg zymosan A into the left hind paw. (**B**) *x*-fold increase (mean ± SD, $n = 6$ each) in spinal COX-2 mRNA expression. All 3 types of mice exhibited a significant increase in COX-2 mRNA expression at 6 hours after zymosan A injection ($P < 0.05$, ANOVA followed by Scheffe's post hoc test, $n = 4$–6 each). (**C**–**F**) Time course of thermal (**C** and **D**) and mechanical (**E** and **F**) sensitization (mean ± SD, $n = 6$ each) after zymosan A injection. **D** and **F** show the same data as **C** and **E** on a different time scale. Thermal sensitization in $EP2^{-/-}$ mice was significantly different from that in WT mice at all time points from 4 hours after zymosan A injection, and mechanical stimulation in $EP2^{-/-}$ mice was significantly different from that in WT mice at all time points from 6 hours after zymosan A injection. $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA followed by Scheffe's post hoc test).

enhanced GFP (EGFP) specifically in glycinergic neurons under the control of the neuronal glycine transporter type 2 (GlyT2) promoter (26). Both glycinergic and nonglycinergic neurons received glycinergic input of similar amplitude (451 ± 104 pA and 498 ± 147 pA for EGFP-positive and -negative neurons, respectively, $n = 10$ each) and with similar kinetics (rinse time = 1.69 ± 0.19 and 1.96 ± 0.16 milliseconds, and decay time = 17.8 ± 3.1 and 14.7 ± 2.0 milliseconds, in EGFP-positive and -negative neurons, respectively) (Figure 7, A and B). However, significant (at least 10%) inhibition of glycinergic IPSCs by PGE_2 occurred much more frequently in nonglycinergic neurons (8 of 10), presumed to be glutamatergic, than in glycinergic neurons (2 of 10, $P < 0.01$, χ^2 test).

Discussion

Although PGE_2 has long been recognized as a lipid mediator produced in many organs throughout the body in response to a variety of physiological and pathological stimuli, the contribution of individual EP receptor subtypes to defined functions of PGE_2 has long remained elusive. This question is of major therapeutic relevance, because the majority of desired and unwanted effects of COX inhibitors are due to the blockade of PGE_2 production. Both the generation of genetically modified mice lacking the different EP receptor subtypes and the development of EP receptor subtype–specific ligands have provided new insights (for a review see ref. 27). Using a genetic approach, we now have identified the EP2 receptor subtype as a key signaling element in spinal inflammatory hyperalgesia. The present study thus adds to the growing evidence that the targeting of individual PG receptor subtypes permits the separation of desired and unwanted effects of NSAIDs (28).

Moreover, our results attribute to PGE_2 a dominant role in spinal pain sensitization. Although other PGs have been implicated in this process (e.g., refs. 29, 30), our findings correlate well with the selective upregulation of microsomal PGE_2 synthase-1 (mPGES-1) in the spinal cord after peripheral inflammation (31) and diminished nociceptive responses in mPGES-1–deficient mice (32). More controversial is the contribution of the different EP receptor subtypes to pain sensitization in the spinal cord. Indeed, several reports suggested a critical role of spinal EP1 receptors in PGE_2-mediated pain sensitization. Although expression of EP1 receptors has not been demonstrated for intrinsic spinal cord neurons (33), they are found on the central (spinal) terminals of primary nociceptive nerve fibers (34), where they might facilitate the release of excitatory neurotransmitters (35). However, most of the behavioral studies rely on the intrathecal injection of the EP1 receptor antagonists (e.g., ONO-8711; refs. 12, 36). Their specificity in these in vivo experiments is difficult to judge, because the actual concentration in the spinal cord tissue is unknown in these studies.

Our results provide new insights into the neurophysiological basis of spinal inflammatory pain sensitization. We have previously demonstrated that PGE_2 reduces inhibitory (strychnine-sensitive) glycinergic neurotransmission in the spinal cord dorsal horn (20, 21). Two sets of experiments now demonstrate that activation of this pathway by EP2 receptors is the dominant mechanism of spinal

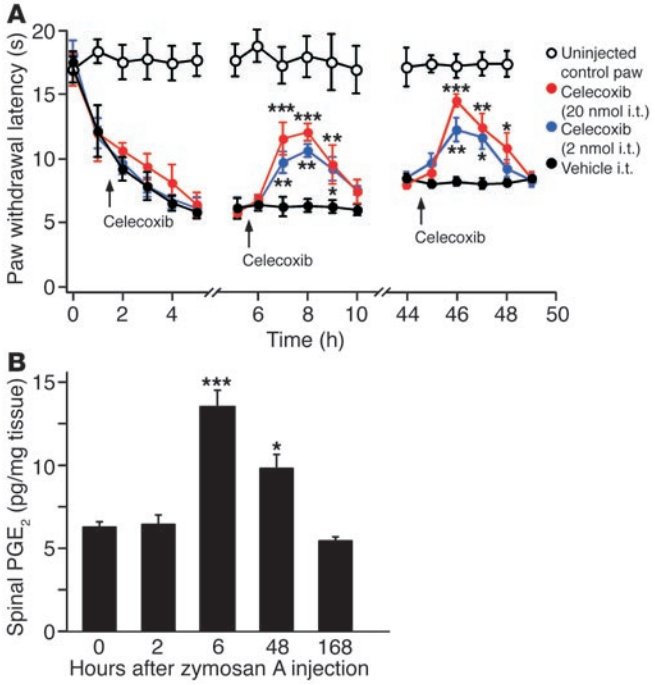

Figure 5
Contribution of COX-2–derived PGs to spinal inflammatory pain sensitization. (**A**) Paw withdrawal latencies (mean ± SD, $n = 6$ each) versus time after zymosan A injection (0.06 mg s.c. into the left hind paw). At the times indicated (arrows), 2 or 20 nmol celecoxib was injected intrathecally ($n = 6$–7). (**B**) Spinal PGE_2 concentrations (mean ± SEM) after zymosan A injection ($n = 4$–5). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

inflammatory pain sensitization. First, EP2$^{-/-}$ mice were protected from spinal hyperalgesia elicited both by intrathecal PGE_2 injection and by zymosan A–induced peripheral inflammation, and second, inhibition of glycinergic neurotransmission by PGE_2 was absent in EP2$^{-/-}$ mice. In these respects the phenotype of the EP2$^{-/-}$ mice very much resembles that of mice deficient in the glycine receptor α3 subunit (21), which also lack PGE_2-mediated inhibition of glycinergic neurotransmission. Our experiments with the BAC transgenic mice expressing EGFP in glycinergic interneurons have now demonstrated that the inhibitory effect of PGE_2 on glycinergic synaptic inhibition is restricted to nonglycinergic interneurons. The majority of the neurons are most likely excitatory and use L-glutamate as their fast neurotransmitter. PGE_2 thus preferentially impairs the glycinergic inhibitory control of excitatory interneurons. This promotes the propagation of nociceptive signals through the spinal cord to higher CNS areas and thereby gives rise to the development of spinal hyperalgesia. This mechanism may also explain why COX inhibitors are primarily antihyperalgesic agents and do not exert a general analgesic activity as opioids do.

Experiments with the COX-2–selective inhibitor celecoxib indicate that the PGE_2 responsible for spinal EP2 receptor activation comes from COX-2, which is induced in the spinal cord dorsal horn in response to peripheral inflammation (this study and refs. 17, 37). The time course of the analgesic action of celecoxib, with only very little or no analgesic effect during early hyperalgesia (2–4 hours after zymosan A injection) but pronounced analgesia at later stages, nicely corresponds to the time course of spinal PGE_2 production and perfectly matches the fast recovery from inflammatory hyperalgesia in EP2$^{-/-}$ mice (compare Figures 4 and 5). The time course of spinal PGE_2 production also explains why PGE_2 caused very fast responses after intrathecal injection and in the electrophysiological experiments, while EP2 receptor–dependent pain sensitization required more than 4 hours for full expression after zymosan A injection. Our experiments hence demonstrate that PGE_2-dependent changes in the spinal processing of nociceptive input develop within a few hours and become the dominant source of inflammatory hyperalgesia, which can significantly outlast the peripheral symptoms of inflammation (compare Figures 4A and 4B). Other, EP2 receptor–independent mechanisms of inflammatory hyperalgesia appear to be of major relevance only early in the development of inflammation and are most likely peripheral in nature. They probably include the activation of EP1 or prostacyclin (IP) receptors, as demonstrated by the deficits in peripheral inflammatory pain sensitization observed in EP1 receptor– or IP receptor–deficient mice (8, 10). It is apparent from our study that neither EP2 nor EP3 receptors contribute to paw swelling or peripheral thermal sensitization. The specific loss of spinal inflammatory pain sensitization in EP2$^{-/-}$ mice correlates well with the expression profile of EP receptors in the nervous system. While in intrinsic spinal cord neurons EP receptor expression is best documented for the EP2 subtype (22), EP1, EP3, and EP4 are predominant in primary nociceptive afferents (34). Nevertheless, part of the peripheral component of mechanical sensitization is apparently mediated by EP2 receptors and may originate from facilitation of tetrodotoxin-resistant Na^+ channels (14), which are of particular relevance to the sensation of noxious mechanical stimuli (38).

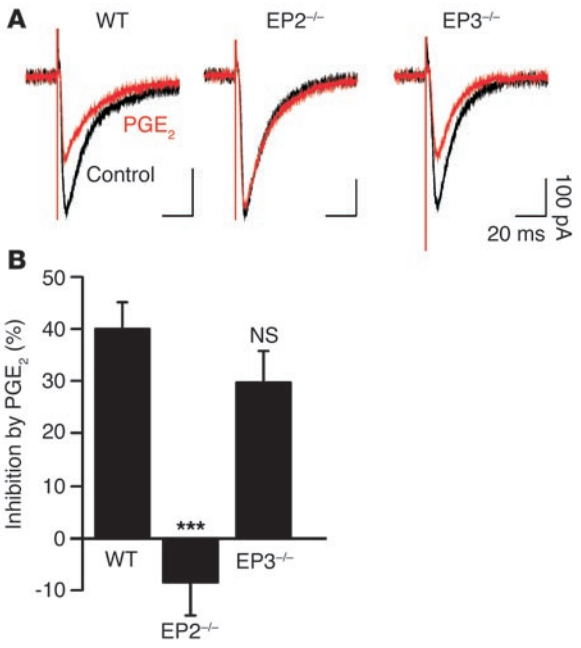

Figure 6
Effects of PGE_2 on glycinergic inhibitory neurotransmission in the spinal cord dorsal horn. (**A**) Averages of 10 glycinergic IPSCs recorded from superficial spinal cord dorsal horn neurons before (black) and during (red) application of PGE_2 (1 μM) in WT, EP2$^{-/-}$, and EP3$^{-/-}$ mice. (**B**) Statistical analysis (mean ± SEM, $n = 8$–10) of IPSC inhibition by PGE_2. Analysis includes PGE_2-responsive and nonresponsive neurons. $^{***}P \leq 0.001$.

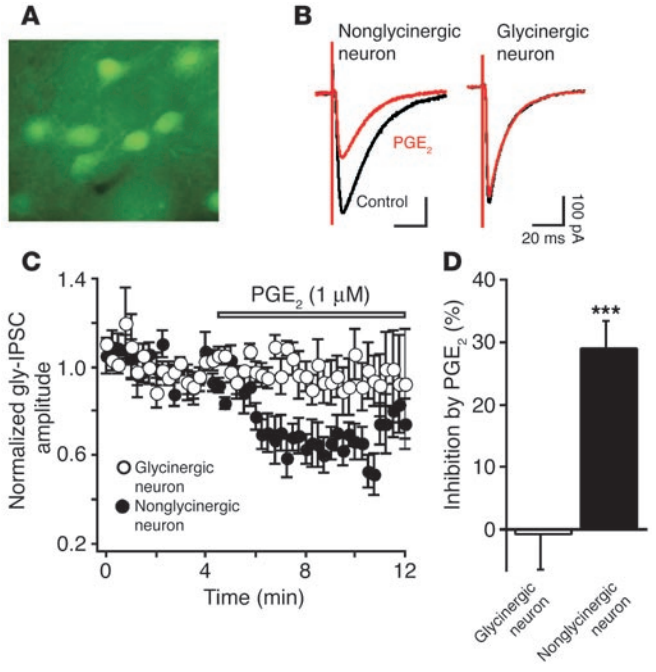

Figure 7
Whole-cell patch-clamp recordings from visually identified glycinergic neurons in the superficial mouse spinal cord dorsal horn. (**A**) EGFP-expressing neurons in the superficial layers of the mouse spinal cord dorsal horn, visualized in a 250-μm-thick slice prepared from a transgenic mouse expressing EGFP in glycinergic neurons under the control of the GlyT2 promoter. (**B**) Averages of 10 consecutive glycinergic IPSCs recorded from an EGFP/GlyT2-negative and an EGFP/GlyT2-positive neuron under control conditions, in the presence of PGE_2 (1 μM), and after its removal. (**C**) Time course of inhibition of glycinergic IPSCs by PGE_2 in EGFP-positive and -negative neurons. (**D**) Average inhibition (mean ± SEM) of glycinergic IPSCs by PGE_2 in EGFP-positive and -negative neurons (n = 10 each). Both PGE_2-responsive and nonresponsive neurons were included for the statistics. ***P < 0.001 (unpaired t test).

In summary, our results attribute to spinal EP2 receptors a dominant role in the generation of inflammatory pain. They point to a novel, more specific, and probably better-tolerated analgesic strategy employing EP2 receptor antagonists as centrally acting, nonopioidergic, antihyperalgesic agents.

Methods

Mice. Behavioral and electrophysiological experiments were performed in EP2 (*ptger2*) and EP3 (*ptger3*) receptor-deficient mice ($EP2^{-/-}$ and $EP3^{-/-}$ mice) (39, 40), which had been backcrossed to the C57BL/6 background for at least 10 generations, and in the corresponding WT mice (C57BL/6). Electrophysiological experiments were in addition performed in BAC transgenic mice expressing EGFP under the control of the GlyT2 (*slc6a5*) promoter in glycinergic interneurons (26). The genotype of all mice analyzed was verified by PCR as described previously (39, 40).

Behavioral testing. Six- to eight-week-old male mice were used for behavioral testing. Mice were kept in the test cages for 1 day to allow accommodation. On day 2, each mouse was tested several times to obtain baseline paw withdrawal latencies and mechanical stimulus-response curves. Paw withdrawal latencies upon exposure to defined radiant heat stimuli were measured using a commercially available apparatus (plantar test; Ugo Basile Biological Research Apparatus Co.). Mechanical sensitivity was determined using von Frey filaments and scored 0, no response; 1, paw withdrawal; or 2, immediate flinching of the stimulated paw (41). Three independent measurements were averaged, and a normalized response score (0–100%) was calculated. Separate groups of mice were used for thermal and mechanical testing. In all behavioral experiments, the observer was blind to the genotype of the mice. For intrathecal injections, PGE_2 was dissolved in 1% ethanol, 99% artificial cerebrospinal fluid (ACSF), and injected in a total volume of 2 μl. Intrathecal injections were made into the lower lumbar spinal canal using a Hamilton Co. syringe (for details see ref. 42). For s.c. injections, PGE_2 was dissolved in 0.1% DMSO, 99.9% PBS, and injected in a total volume of 5 μl. In both cases vehicle did not cause nociceptive sensitization. Because of its poor solubility, celecoxib was dissolved in 20% DMSO, 80% ACSF, and injected in a total volume of 10 μl. Zymosan A (0.06 mg in 20 μl PBS) was injected into the plantar side of the left hind paw. All behavioral experiments were performed in an air-conditioned room (22°C). After the tests the mice were killed by CO_2 inhalation. All animal experiments were performed in accordance with the institutional guidelines of the University of Erlangen-Nürnberg and of the European Communities Council Directive (86/609/EEC) and were approved by the animal welfare committee of the Regierung von Unterfranken. Permission was obtained from the local government (Regierung von Mittelfranken, reference no. 621-2531.31-17/03).

Quantification of COX-2 mRNA. Mice were killed by decapitation, and tissue samples of the spinal cord segment L4 were snap-frozen in 800 μl of lysis buffer (QIAGEN GmbH) and stored at –70°C. After homogenization, RNA was isolated using an RNeasy kit (QIAGEN GmbH). Real-time RT-PCR was used to quantify actin and COX-2 mRNA. TaqMan probes used were as follows: actin, 5′-(6FAM)TATGCTC(TAMRA)TCCCTCAC GCCATCCTGCT-3′; COX-2, 5′-(6FAM)CTACCATGGTC(TAMRA)TCC CCAAAGATAGCATCA-3′. Primers used were as follows: actin, forward 5′-TCACCCACACTGTGCCCATCTACGA-3′, reverse 5′-GGATGCCACAG-GATTCCATACCCA-3′; COX-2, forward 5′-TTTGTTGAGTCATTCAC-CAGACAGAT-3′, reverse 5′-CAGTATTGAGGAGAACAGATGGGATT-3′ (for PCR conditions see ref. 25).

PGE_2 measurements. PGE_2 measurements were taken using the Correlate EIA Kit (Assay Designs Inc.). The thoracolumbar segment of the spinal cord was removed and transferred into 99.5% methanol (500 μl), stored for 24 hours at –20°C, and then shaken for 2 hours at room temperature. The spinal cord tissue was then removed, and the methanol was evaporated. The remaining pellet was dissolved in 100 μl of enzyme immunoassay buffer. Measurement was done using an ELISA reader (DIAS microplate reader; Dynatech Laboratories) with an absorbance maximum at 405 nm.

Electrophysiological recordings in spinal cord slices. Ten- to fourteen-day-old mice of both sexes were killed under ether narcosis by decapitation (for details see ref. 20). Transverse slices, 250 μm thick, of the lumbar spinal cord, were prepared as described previously. Whole-cell patch-clamp recordings were performed from neurons located in lamina II of the spinal cord dorsal horn. Neurons were visually identified using the infrared gradient contrast technique coupled to a video microscopy system. Slices were continuously superfused with external solution, which contained (in mM) 125 NaCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose (pH 7.30, 315 mosm/l) and was bubbled with 95% O_2, 5% CO_2. Patch pipettes (4–5 MΩ) were filled with internal solution containing (in mM) 130 K-gluconate, 20 KCl, 2 $MgCl_2$, 0.05 EGTA, 3 Na-ATP, 0.1 Na-GTP, 10 Na-HEPES (pH 7.30). Five millimolar QX-314 was added to the internal solution to block voltage-activated sodium currents. Postsynaptic current responses were elicited at a frequency of 1 per 15 seconds by extracellular electrical field stimulation (100 microseconds, 3–10 V) and recorded at room temperature and at a holding potential of –80 mV. Glycinergic IPSCs were isolated using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM),

D-2-amino-5-phosphonovaleric acid (D-APV; 50 μM), and bicuculline (10 μM). Short hyperpolarizing voltage steps to –90 mV were applied in 1-minute intervals to monitor input and access resistance. PGE_2 (1 μM) was applied by bath perfusion at a rate of 1–2 ml/min.

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