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Spinal prostaglandin E receptors of the EP2 subtype and the glycine receptor α3 subunit, which mediate central inflammatory hyperalgesia, do not contribute to pain after peripheral nerve injury or formalin injection

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Abstract

Inflammation, peripheral nerve injury and chemical irritants can cause central sensitization in pain pathways. Prostaglandins produced in the CNS induce central sensitization during inflammation mainly by relieving nociceptive neurons from glycinergic inhibition. We have recently identified spinal prostaglandin E receptors of the EP2 subtype (EP2 receptors) and the glycine receptor α3 subunit (GlyRα3) as signal transduction elements involved in the generation of central inflammatory hyperalgesia. It is however still unknown to what extent inhibition of glycine receptors by PGE_2 contributes to neuropathic or chemically induced pain. To address this question, we have analyzed mice deficient in the EP2 receptor ($EP2^{-/-}$ mice) or in the GlyRα3 subunit ($GlyR\alpha3^{-/-}$ mice) using the chronic constriction injury (CCI) model of neuropathic pain and the formalin test. We found that $EP2^{-/-}$ mice and $GlyR\alpha3^{-/-}$ mice develop thermal and mechanical hyperalgesia in the CCI model indistinguishable from that seen in wild-type mice. In the formalin test, $EP2^{-/-}$ mice, but not $GlyR\alpha3^{-/-}$ mice, exhibited reduced nocifensive behavior. The lack of a phenotype in $GlyR\alpha3^{-/-}$ mice together with the absence of a facilitating effect of intrathecal PGE_2 on formalin-induced nociception in wild-type mice suggests that peripheral rather than spinal EP2 receptors are involved. These results indicate that inhibition of glycinergic neurotransmission by EP2 receptor activation does not contribute to pain following peripheral nerve injury or chemical irritation with formalin. Our results thus provide further evidence that inflammatory hyperalgesia and neuropathic pain involve different mechanisms of central sensitization.

Keywords: Neuropathic pain; Inflammatory pain; Formalin test; Hyperalgesia; PGE2; Prostaglandin E2; Glycine; Spinal cord

1. Introduction

Inflammatory and neuropathic diseases are frequently accompanied by exaggerated pain sensitivity. In addition to peripheral processes, central sensitization

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significantly contributes to this phenomenon (Woolf, 1983). Spinal prostaglandin E_2 (PGE_2) is largely responsible for central sensitization following peripheral inflammation. Cyclooxygenase (COX)-2 (Beiche et al., 1996; Samad et al., 2001) and microsomal prostaglandin E synthase (Guay et al., 2004) are induced in the spinal cord in response to peripheral inflammation. PGE_2 produced by these enzymes inhibits glycinergic neurotransmission (Ahmadi et al., 2002) and subsequently relieves dorsal horn nociceptive neurons from the inhibitory control by glycinergic neurons (Reinold et al., 2005). This disinhibition results from an activation of PGE_2 receptors of the EP2 subtype (EP2 receptor) and a subsequent protein-kinase A-dependent block of glycine receptors containing the α3 subunit (GlyRα3) (Harvey et al., 2004). Mice deficient in either the EP2 receptor or in GlyRα3 ($EP2^{-/-}$ and $GlyR\alpha3^{-/-}$ mice) almost completely lack the pronociceptive effects of spinal PGE_2 and recover much faster from inflammatory hyperalgesia than the corresponding wild-type mice (Harvey et al., 2004; Reinold et al., 2005; Zeilhofer, 2005b).

Relief from inhibition also contributes to neuropathic pain after peripheral nerve injury (Moore et al., 2002; Coull et al., 2003; Coull et al., 2005). However, the role of prostaglandins in this process is controversial and no data are available on a possible contribution of PGE_2-mediated inhibition of glycine receptors to neuropathic pain. Nerve damage up-regulates COX-1 and COX-2 and various types of EP receptors in the injured nerve and in the spinal cord (Zhu and Eisenach, 2000; Ma and Eisenach, 2003a; Ma and Eisenach, 2003b; O'Rielly and Loomis, 2006). Brush-evoked increases in spinal PGE_2 and an increased sensitivity to PGE_2 of the spinal cord following nerve injury (Hefferan et al., 2003a,b) also support a role of PGE_2. Other groups however have found no major induction of COX-2 in response to nerve injury (e.g., Broom et al., 2004) and similar controversies exist regarding possible antinociceptive effects of COX inhibitors in neuropathic pain. While Broom et al. (2004) and Schäfers et al. (2004) found only a moderate antinociceptive action of either selective or non-selective inhibitors, Hefferan et al. (2003b) reported significant antinociception by non-selective COX inhibitors.

A similar controversy exists for the formalin test. Several studies found significant antinociceptive activity of non-selective COX inhibitors (Malmberg and Yaksh, 1992; Dirig et al., 1997; Euchenhofer et al., 1998; Tegeder et al., 2001). Others however reported that PGE_2 neither facilitates formalin-induced pain nor reverses antinociception induced by COX inhibitors (Gühring et al., 2002; Ates et al., 2003).

$EP2^{-/-}$ and $GlyR\alpha3^{-/-}$ mice allowed us now to investigate whether EP2 receptor-dependent inhibition of glycinergic neurotransmission contributes to hyperalgesia following peripheral nerve injury or subcutaneous injection of chemical irritants. Our results demonstrate that this pathway neither contributes to pain sensitization in the chronic constriction injury (CCI) model of neuropathic pain nor to nocifensive behavior in the formalin test. They further indicate that inflammatory and neuropathic diseases as well as chemically induced pain involve different mechanisms of central sensitization.

2. Materials and methods

2.1. Mice

Behavioral experiments were performed in EP2 (*ptger2*) receptor-deficient mice ($EP2^{-/-}$ mice; Hizaki et al., 1999) and in mice lacking the α3 subunit of inhibitory glycine receptors (*glra3*) ($GlyR\alpha3^{-/-}$; Harvey et al., 2004), which had been back-crossed to the C57/Bl6 background for 10 and 6 generations, respectively, and in the corresponding wild-type mice (C57/Bl6). $EP2^{-/-}$ mice were kindly provided by Dr. Shuh Narumiya, Department of Pharmacology, Kyoto University, Japan. $GlyR\alpha3^{-/-}$ mice were from Dr. Ulrike Müller, Max-Planck-Institut für Hirnforschung, Frankfurt, Germany. The genotypes of all mice analyzed were verified by PCR as described previously (Hizaki et al., 1999; Harvey et al., 2004). 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). Permission to conduct the presented experiments was obtained from the local government (Regierung von Mittelfranken, Ref. 621-2531.31-17/03). In all experiments the observer was blinded with respect to the genotype of the mice.

2.2. Chronic constriction injury (Bennett and Xie, 1988)

Mice were anesthetized with isoflurane. A unilateral constriction injury of the left sciatic nerve just proximal to the trifurcation was performed with three loose ligatures using a 5–0 silk thread. The development of thermal and mechanical hyperalgesia was assessed as described previously (Depner et al., 2003; Reinold et al., 2005). Briefly, paw withdrawal latencies upon exposure to a defined radiant heat stimulus were determined using a commercially available apparatus (plantar test; Ugo Basile Biological Research Apparatus Co.). Mechanical sensitivity was tested with calibrated von-Frey filaments. A reaction score (0, no response; 1, slow response; 2, immediate withdrawal of the paw) was determined in three independent measurements and normalized to 0–100% (for details, see Depner et al., 2003). Sham-operated mice, which underwent the same surgical procedure but without nerve ligatures, served as controls.

2.3. Formalin test (Dubuisson and Dennis, 1977)

Formalin (20 μl, 0.5% or 5%) was injected subcutaneously into the dorsal surface of the left hind paw. Flinches of the injected paw were counted for 60 min in 1 min intervals starting immediately after formalin injection. Determining the time spent licking or biting the injected paw gave similar but, in our hands, less reproducible results. PGE_2 (dissolved in 99% artificial cerebrospinal fluid/1% ethanol) was injected intrathecally in a total volume of 2 μl 10 min prior to formalin injection using a

Hamilton syringe (for details, see Ahmadi et al., 2001). A small amount of black ink was included in the injection volume to allow visual verification of proper intrathecal injection after completion of the experiment. Two out of 27 mice were excluded from the analysis, because no black ink was visible on top of the lumbar spinal cord after laminectomy.

3. Results

3.1. Chronic constriction injury model of neuropathic pain

The contribution of PGE_2-mediated blockade of inhibitory glycine receptors to neuropathic pain after peripheral nerve damage was tested using the chronic constriction injury (CCI) model. Three loose ligatures of the left sciatic nerve were made in $EP2^{-/-}$ mice and $GlyR\alpha3^{-/-}$ mice, which both lack the pronociceptive effects of spinal PGE_2, and in the respective wild-type mice. As reported previously (Reinold et al., 2005), wild-type mice and $EP2^{-/-}$ mice did not differ significantly in their sensitivities to thermal and mechanical stimuli ($P > 0.93$ and $P > 0.16$, for thermal and mechanical sensitivity at 16 mN von-Frey filament force; t-test; $n = 5$, each; compare also baseline values in Fig. 1A–C). Following nerve ligation, wild-type mice developed progressive thermal and mechanical hyperalgesia in the affected paw. Sensitization reached its maximum between 5 and 7 days after surgery and then remained constant for the rest of the experiment, which lasted 3 weeks in total. At day 7 after surgery, paw withdrawal latencies upon stimulation with a defined radiant heat stimulus had decreased from 16.2 ± 0.8 s at baseline to 6.6 ± 0.4 s ($n = 5$). Mechanical sensitization was measured in response to stimulation with calibrated von-Frey filaments. At a stimulation strength of 16 mN reaction scores increased from $35 \pm 4\%$ ($n = 5$) at baseline to $85 \pm 7\%$ seven days after surgery (Fig. 1B). Similar mechanical sensitization was found over the whole range of stimulation strengths tested (1–64 mN; Fig. 1C). Minor thermal and mechanical sensitization was also seen in the contralateral (non-operated) paw (data not shown). Sham-operated mice, which had undergone the same surgery but without nerve ligatures, developed only minor thermal and mechanical hyperalgesia (Fig. 1A and B). Differences in thermal and mechanical sensitivity between wild-type mice and $EP2^{-/-}$ mice remained below statistical significance for all time points tested (Fig. 1).

Pain sensitization in $GlyR\alpha3^{-/-}$ mice after CCI was assessed using the same parameters as those described above. Again no significant differences in sensitization were observed in $GlyR\alpha3^{-/-}$ as compared to wild-type mice. Baseline latencies upon exposure to noxious heat and mechanical reaction scores were almost identical in wild-type and $GlyR\alpha3^{-/-}$ mice (compare Fig. 2A–C). Following surgery, $GlyR\alpha3^{-/-}$ and wild-type mice developed nearly identical thermal and mechanical

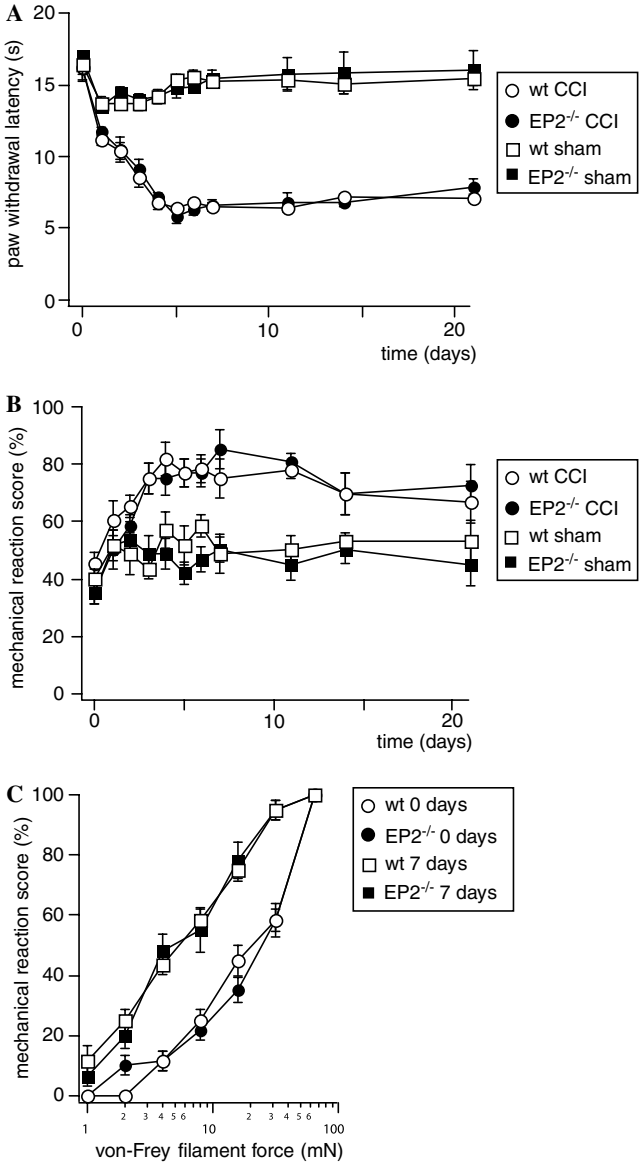

Fig. 1. EP2 receptor-deficient mice in the chronic constriction injury model of neuropathic pain. (A) Paw withdrawal latencies (mean ± SEM) in response to stimulation with a defined radiant heat stimulus versus time after surgery. In wild-type mice (wt, ○, □) and $EP2^{-/-}$ mice (●, ■) thermal sensitization was significant versus baseline for all time points tested after nerve ligation ($P \leqslant 0.05$; ANOVA followed by Dunnett's post hoc test). Sensitization in CCI mice was also significant versus sham-operated control mice (□, ■) at all time points tested ($P \leqslant 0.05$ – $P \leqslant 0.001$, ANOVA followed by Fisher's post hoc test). (B) Nociceptive reaction scores (mean ± SEM) upon stimulation with a calibrated 16 mN von-Frey filament versus time after surgery. Mechanical sensitization became statistically significant versus baseline ($P \leqslant 0.05$, ANOVA followed by Dunnett's post hoc test) 2 days after nerve ligation in both wild-type mice (○) and $EP2^{-/-}$ mice (●). Sensitization in CCI mice of both genotypes also became significantly different from sham-operated control mice (□, ■) at 3 days after surgery ($P \leqslant 0.05$ – $P \leqslant 0.01$, ANOVA followed by Fisher's post hoc test). (C) Mechanical stimulus response curves (mean ± SEM) in wild-type (○) and $EP2^{-/-}$ mice (●) before surgery and 7 days after, when maximum sensitization had developed. $n = 5$ mice/group and time point.

hyperalgesia (Fig. 2A and B). Mechanical sensitization occurred through the entire range of stimulation strengths tested (Fig. 2C).

3.2. Formalin test

A possible contribution of EP2 receptor-dependent inhibition of glycine receptors to chemically induced pain was assessed using the formalin test (Fig. 3). Wild-type mice, EP2$^{-/-}$ and GlyRα3$^{-/-}$ mice exhibited a bi-phasic flinching behavior after subcutaneous injection of formalin into the left hind paw. In phase I (0–10 min), all three types of mice showed similar nocifensive reactions. In phase II (20–60 min), EP2$^{-/-}$ mice exhibited a significant reduction in the number of flinches suggesting a possible involvement of EP2 receptors in central sensitization in the formalin test. However, a similar reduction in the number of flinches was not seen in GlyRα3$^{-/-}$ mice. Thus, the absence of PGE_2-mediated inhibition of GlyRα3 inhibition cannot account for the reduction of formalin-induced nocifensive behavior seen in EP2$^{-/-}$ mice.

Alternatively, spinal EP2 receptors might affect targets other than GlyRα3 in the formalin test. To test this hypothesis, we investigated the effect of intrathecal PGE_2 injection on formalin-induced nocifensive behavior in wild-type mice (Fig. 4). In these experiments we injected the same dose of PGE_2 (0.2 nmol/mouse) that was previously shown to elicit significant thermal and mechanical hyperalgesia lasting for more than 60 min (Harvey et al., 2004; Reinold et al., 2005). In a first set of experiments we reproduced the pronociceptive effect of intrathecal PGE_2 in naive wild-type mice. As expected, paw withdrawal latencies upon exposure to noxious heat decreased from 17.2 ± 0.3 s (mean ± SEM, $n = 5$) under control conditions to 9.92 ± 0.4 s ($P \leqslant 0.001$, paired t-test) 30 min after PGE_2 injection, while vehicle injection was without significant effect (baseline 16.9 ± 0.3 s versus 16.0 ± 0.3 s, $n = 5$, $P > 0.05$, paired t-test). No spontaneous "flinching" behavior was observed in PGE_2-treated mice (data not shown). Although i.t. PGE_2 caused significant thermal hyperalgesia, it had no effect on the number of formalin-induced flinches. To exclude the possibility that spinal EP receptors were already saturated by endogenous PGE_2 we performed additional experiments with a sub-saturating dose of formalin (20 µl, 0.5%). In these experiments the number of flinches evoked by formalin was strongly reduced, but intrathecal PGE_2 was still without effect.

4. Discussion

Our results demonstrate that neither EP2 receptors nor the inhibition of glycinergic neurotransmission following EP2 receptor activation is necessary for the development of neuropathic pain after peripheral nerve injury in the CCI model. Although our study *per se* does

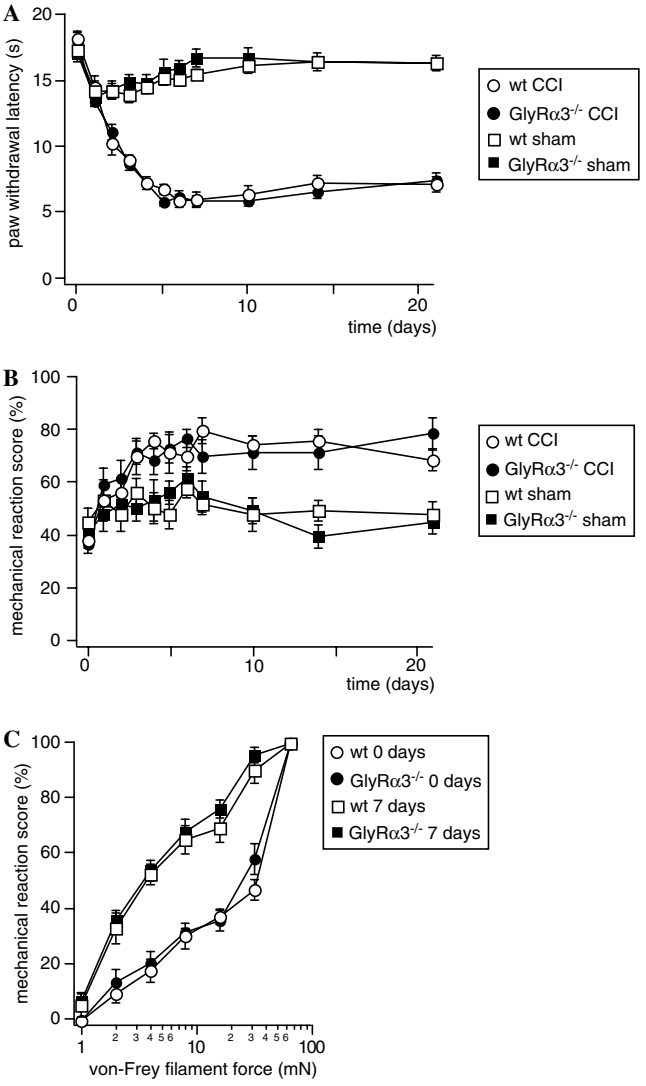

Fig. 2. GlyRα3$^{-/-}$ mice in the chronic constriction injury model of neuropathic pain. Same experimental conditions as Fig. 1. (A) Thermal sensitization. Paw withdrawal latencies (mean ± SEM, $n = 6$, each) versus time after surgery in wild-type mice (wt, ○,□) and GlyRα3$^{-/-}$ mice (●,■). Nerve-ligated mice (○,●), sham-operated (□,■). Thermal sensitization compared with baseline values became statistically significant at day 1 after surgery both in wild-type and GlyRα3$^{-/-}$ mice ($P \leqslant 0.05$; ANOVA followed by Dunnett's post hoc test). Differences in paw withdrawal latencies between nerve-ligated mice and sham-operated mice were statistically significant from day 2 after surgery through the end of the experiment (day 21) for both wild-type and GlyRα3$^{-/-}$ mice ($P \leqslant 0.05$ – $P \leqslant 0.001$, ANOVA followed by Fisher's post hoc test). (B) Mechanical sensitization (assessed with a 16 mN von-Frey filament) as compared to baseline sensitivity became statistically significant at day 3 for wild-type mice (○) and at day 2 for GlyRα3$^{-/-}$ mice (●) ($P \leqslant 0.05$, ANOVA followed by Dunnett's post hoc test). Differences between nerve-ligated and sham-operated mice became statistically significant at day 4 after surgery for both wild-type and GlyRα3$^{-/-}$ mice ($P \leqslant 0.05$ – $P \leqslant 0.001$, ANOVA followed by Fisher's post hoc test). (C) Stimulus response curves (mean ± SEM) in naïve wild-type (○) and GlyRα3$^{-/-}$ mice (●) and 7 days after surgery (□, ■; wt and GlyRα3$^{-/-}$ mice) when maximum sensitization had developed. $n = 6$ mice/group and time point.

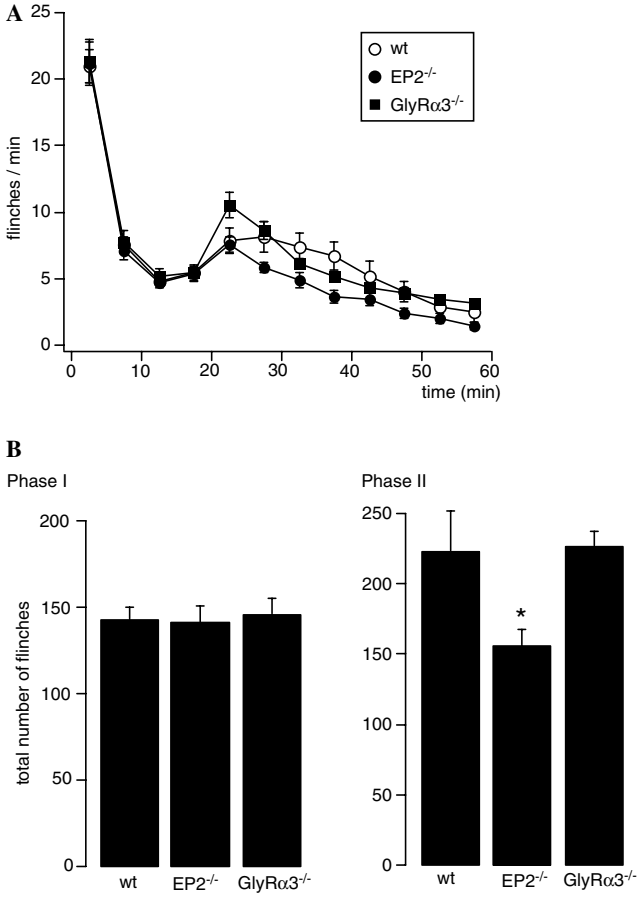

Fig. 3. Formalin test in EP2$^{-/-}$ and GlyRα3$^{-/-}$ mice. (A) number of flinches/minute (mean ± SEM) versus time. ○, wild-type; ●, EP2$^{-/-}$; ■, GlyRα3$^{-/-}$ mice. $n = 10$–12 mice/group. (B) total number of flinches (mean ± SEM) during phase 1 (0–10 min) and phase 2 (20–60 min) of the formalin test. $^{*}P < 0.05$, ANOVA followed by Fisher's post hoc test.

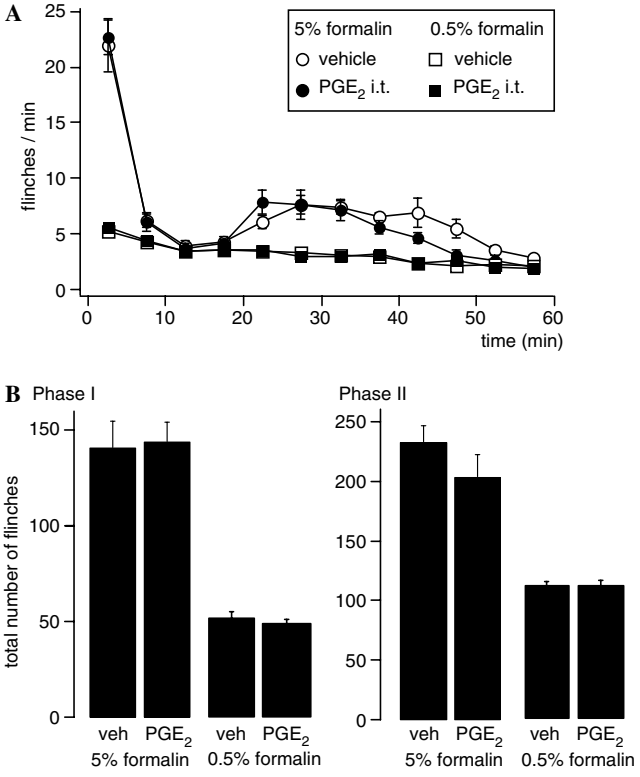

Fig. 4. Intrathecally injected PGE_2 does not affect nocifensive reactions in the formalin test. (A) Number of flinches/min versus time. 20 µl formalin was injected subcutaneously at two different concentrations 0.5% and 5%. PGE_2 was injected intrathecally at a dose of 0.2 nmol/mouse 10 min prior to formalin injection. $n = 10$ mice/group. (B) total number of flinches (mean ± SEM) during phase 1 and phase 2 of the formalin test. ○, 5% formalin/vehicle (1% ethanol); ●, 5% formalin/PGE_2 (0.2 nmol i.t./mouse); □, 0.5% formalin/vehicle; ■, 0.5% formalin, PGE_2.

not exclude a contribution of EP receptors different from EP2 or of other prostaglandins, many lines of evidence argue against this possibility. Both experimental and clinical studies demonstrate that neuropathic pain responds poorly to COX inhibitors. In the CCI model in rats the COX-2-selective inhibitor rofecoxib had a minor effect on thermal hyperalgesia and was completely ineffective against mechanical allodynia (de Vry et al., 2004). Broom et al. (2004) also found no effect of rofecoxib on mechanical sensitization, cold allodynia and pin prick sensitivity in the spared nerve injury model. Another study that used celecoxib and, for comparison, the non-selective COX inhibitor ibuprofen also found only moderate effects of both drugs on thermal and mechanical sensitization (Schäfers et al., 2004). Nevertheless, the lack of a contribution of prostaglandins is somewhat surprising as proinflammatory cytokines, which induce the spinal expression of COX-2 in inflammation (Samad et al., 2001), are also produced in response to peripheral nerve injury (e.g., Lindenlaub and Sommer, 2003). This discrepancy may be explained by the fact that induction of COX-2 after peripheral nerve damage is significantly smaller in magnitude than the induction observed in models of inflammatory pain (Broom et al., 2004) and may therefore not be accompanied by significant increases in spinal PGE_2 (Schäfers et al., 2004). Other studies have suggested that COX-1 inhibition reduces allodynia selectively at early stages in the spinal nerve ligation model (Hefferan et al., 2003a,b). Although inconsistencies in the actual time point or the model used might explain some of the controversies outlined above, EP2$^{-/-}$ and GlyRα3$^{-/-}$ mice tested in the present study did not show a significant reduction in thermal or mechanical sensitivity during three weeks following nerve ligation.

Several candidate pathways have been described *in vitro* that can possibly explain the pronociceptive effects of spinal PGE_2 (for reviews, see Samad et al., 2002; Zeilhofer, 2005a). In addition to inhibiting glycine receptors (Ahmadi et al., 2002; Harvey et al., 2004), PGE_2 has been reported to increase the release of the excitatory neurotransmitter L-glutamate from the spinal terminals of primary afferent nerve fibers (Nishihara

et al., 1995; Minami et al., 1999) and to directly depolarize deep dorsal horn neurons (Baba et al., 2001). While for the latter two pathways the *in vivo* relevance is still largely unknown, the contribution of EP2 receptor-mediated inhibition of glycine receptors is supported by the absence of a pronociceptive effect of intrathecally injected PGE_2 in naïve $EP2^{-/-}$ and $GlyR\alpha3^{-/-}$ mice and by the quick recovery from inflammatory hyperalgesia seen in both types of knock-out mice. Although unlikely, the present experiments do not exclude the induction of other EP receptors in response to peripheral nerve damage, which might activate pathways different from glycine receptor inhibition.

While our data clearly demonstrate a lack of involvement of EP2 and GlyRα3 receptors in neuropathic pain in the CCI model, the results obtained in the formalin test appear to be more complex. $GlyR\alpha3^{-/-}$ mice exhibited nocifensive responses to formalin challenge similar to those observed in wild-type mice indicating that inhibition of GlyRα3 by PGE_2 is not significantly involved in this test. Nevertheless, $EP2^{-/-}$ mice showed a significantly reduced number of flinches in phase II of the formalin test. We cannot exclude that in the formalin test EP2 receptors couple to targets different from GlyRα3, but previous work from our group has shown that the pronociceptive effects of PGE_2 injected intrathecally depend on the presence of GlyRα3 (Harvey et al., 2004). Alternatively, the discrepant phenotypes of $EP2^{-/-}$ and $GlyR\alpha3^{-/-}$ mice in this test may result from an involvement of peripheral rather than spinal EP2 receptors. Direct evidence for an EP2 receptor-dependent nociceptive sensitization of primary sensory neurons is sparse, but RT-PCR studies have detected EP2 receptor mRNA in such neurons (Donaldson et al., 2001; Southall and Vasko, 2001) and Matsumoto et al. (2005) have shown that facilitation of tetrodotoxin-insensitive Na^+ channels in rat nodose ganglia involves EP2 receptors. A similar mechanism in dorsal root ganglion neurons might explain why $EP2^{-/-}$ mice exhibit diminished mechanical sensitization after local injection of PGE_2 into one hind paw (Reinold et al., 2005).

Our observation that intrathecally injected PGE_2 did not increase nocifensive reactions in the formalin test at a dose that reliably evoked pronounced nociceptive sensitization raises doubts about the role of spinal PGE_2 in formalin-induced nociception. In line with this, Minami et al. (2001) reported that $EP1^{-/-}$ and $EP3^{-/-}$ mice exhibit normal nocifensive reactions in the formalin test.

However, non-selective COX inhibitors (Malmberg and Yaksh, 1992), but not COX-2-selective compounds (Dirig et al., 1997), are antinociceptive in the formalin test after intrathecal injection. This might indicate that COX-1 derived prostaglandins other than PGE_2 are involved. However, an alternative and more recently developed hypothesis is that COX inhibitors can produce antinociception by mechanisms independent from the inhibition of prostaglandin production. Although inhibition of PGE_2 production is most likely the dominant antinociceptive mechanism of COX inhibitors in inflammatory pain, other mechanisms may become apparent when the contribution of prostaglandins is less important, e.g., in the formalin test. Gühring et al. (2002) and Ates et al. (2003) reported that they could not reverse the antinociceptive effects of the intrathecal COX inhibitors indomethacin and flurbiprofen by intrathecal PGE_2 in the formalin test. Hence, it is possible that COX inhibitors exert part of their antinociceptive action by shifting arachidonic acid metabolism towards the production of antinociceptive endocannabinoids (as suggested by Gühring et al., 2002) or by reducing their degradation. It has indeed been shown that COX-2 metabolizes the endocannabinoids anandamide and 2-arachidonyl glycerol to PGE_2 ethanolamide and PGH_2 glycerol ester (Yu et al., 1997; Kozak et al., 2000) and recent work from Kim and Alger (2004) suggests that COX-2 inhibitors facilitate the action of endogenous cannabinoids in the hippocampus by blocking their degradation. It is highly conceivable that similar mechanisms in the spinal cord contribute to antinociceptive effects of COX inhibitors in the formalin test.

In summary, our results clearly show that inhibition of glycinergic neurotransmission by PGE_2 does not contribute to neuropathic pain after peripheral nerve injury. Our study thus provides further evidence that inflammatory and neuropathic pain involve different mechanisms of central sensitization. The prominent phenotypes of $EP2^{-/-}$ and $GlyR\alpha3^{-/-}$ mice in models of peripheral inflammation are in strong contrast with the phenotypes observed in the formalin test. Data obtained in the formalin test should therefore be interpreted only with caution when related to inflammatory hyperalgesia.

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