PAIN® 155 (2014) 403–412

www.elsevier.com/locate/pain

Etifoxine analgesia in experimental monoarthritis: A combined action that protects spinal inhibition and limits central inflammatory processes

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ARTICLE INFO

Article history:
Received 8 July 2013
Received in revised form 27 September 2013
Accepted 4 November 2013

Keywords:
BDNF
Central inflammation
COX2
GABA
Glycine
Microglia
Nonbenzodiazepine anxiolytic
PGE2
Spinal pain processing

ABSTRACT

Inflammatory and degenerative diseases of the joint are major causes of chronic pain. Long-lasting pain symptoms are thought to result from a central sensitization of nociceptive circuits. These processes include activation of microglia and spinal disinhibition. Using a monoarthritic rat model of pain, we tried to potentiate neural inhibition by using etifoxine (EFX), a nonbenzodiazepine anxiolytic that acts as an allosteric-positive modulator of gamma-aminobutyric acid type A (GABAA) receptor function. Interestingly, EFX also can bind to the mitochondrial translocator protein (TSPO) complex and stimulate the synthesis of 3α-reduced neurosteroids, the most potent positive allosteric modulator of GABAA receptor function. Here we show that a curative and a preventive treatment with 50 mg/kg of EFX efficiently reduced neuropathic pain symptoms. In the spinal cord, EFX analgesia was accompanied by a reduction in microglial activation and in the levels of proinflammatory mediators. Using electrophysiological tools, we found that EFX treatment not only amplified spinal GABAergic inhibition, but also prevented prostaglandin E2–induced glycinergic disinhibition and restored a "normal" spinal pain processing. Because EFX is already distributed in several countries under the trade name of Stresam for its anxiolytic actions in humans, new clinical trials are now required to further extend its therapeutic indications as pain killer.

1. Introduction

Inflammatory and degenerative diseases of joints are major causes of chronic pain [6]. To better understand the pathophysiological mechanisms associated with joint pain and to design new therapeutic strategies, chronic inflammatory pain resulting from a unilateral intra-articular knee injection of complete Freund's adjuvant (CFA) has been well characterized [8]. CFA induces several plastic changes in the nociceptive pathways, including sensitization of joint nociceptors [18,42] and of central neurons [31,43]. These processes are thought to account for the expression of spontaneous pain behaviors and pain symptoms [41].

Proinflammatory mediators, overproduced at the injured site [37] and in the spinal cord, contribute in large part to the early phase and maintenance of sensitization processes [38,46,50]. For example, neutralization of tumor necrosis factor α (TNFα), directly at the joint or in the spinal cord of monoarthritic rats, produces significant antinociception [4,5]. Increased prostaglandin E2 (PGE2) synthesis and release has been demonstrated clearly in the spinal cord of rats suffering from monoarthritic pain [51] as a result of overexpression of the inducible PGE2-synthesizing enzyme cyclooxygenase type 2 [16]. Agreeing with these findings is research showing that intrathecal application of PGE2 produces significant excitation of spinal cord neurons in naive rats [49], and increases pain symptoms when injected in vivo [29,30,48]. Further studies have revealed that PGE2-induced central sensitization and pain symptoms result from a loss of glycine receptor–mediated synaptic inhibition [1], achieved by the activation of prostanoid EP2 receptors [35] and by hyperphosphorylation of α3 subunit–containing glycine receptors (GlyRs), expressed by spinal neurons in the most superficial dorsal horn layers [21]. If proinflammatory cytokines can be released by activated microglia and astrocytes within the nociceptive system, they also can secrete trophic factors, such as brain-derived neurotrophic factor (BDNF). Microglial BDNF recently was found to be involved in the downregulation of KCC2 potassium chloride exporters in the spinal cord of neuropathic rats

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0304-3959/$36.00
http://dx.doi.org/10.1016/j.pain.2013.11.003

[12]. KCC2 downregulation also has been found to deeply alter chloride gradients in nociceptive-specific spinal neurons and to reduce inhibitory chloride currents mediated by gamma-aminobutyric acid type A (GABAA) receptors (GABAARs) and GlyRs [13].

In summary, preventing PGE2-induced glycinergic disinhibition in the spinal cord and BDNF-mediated downregulation of KCC2 expression is an interesting option to limit spinal nociceptive processing and pain symptoms. Another strategy could consist of potentiating the remaining GABAAR-dependent inhibitory controls using positive allosteric modulators such as etifoxine (EFX), a non-benzodiazepine anxiolytic [28,32,44] binding preferentially to β2 subunits [19]. EFX also stimulates the synthesis of neuroactive steroids [39] such as the allopregnanolone, a 3α-reduced neurosteroid (3αNS) with potent analgesic properties [9]. In a chemotherapy-induced neuropathic pain model, EFX treatment fully alleviated pain symptoms [2] and these pain symptoms never reappeared after the end of the treatment. This observation is unlikely to be solely explained by direct (allosteric) and indirect action (production of endogenous allosteric modulator: 3αNS) on GABAARs but rather due to long-term changes in gene expression and possible local chloride homeostasis.

We tested this hypothesis on inflammatory-driven pain symptoms in rats using the well-characterized CFA-induced monoarthritic model of pain. We analyzed the possible effects of EFX on the central inflammatory response and its consequences on spinal inhibition.

2. Methods

2.1. Animals

In this study, male Sprague-Dawley rats 250 to 350 g (Janvier, Le Genest St. Isle, France) were housed in groups of 3 under standard conditions (room temperature: 22°C; 12/12-hour light–dark cycle) with ad libitum access to food and water. All experiments were conducted in conformity with the recommendations of the European Committee Council Direction of September 22, 2010 (2010/63/EU). Procedures were positively evaluated by the regional ethical committee, and experiments were conducted with an official authorization for animal experimentation from the French Department of Agriculture (License 67-116 to P.P.).

2.2. Behavioral testing

All animals were habituated to the room and to the tests at least 1 week before starting the experiments. Mechanical nociceptive thresholds were measured using a calibrated forceps (Bioseb, Vitrolles, France) as previously described [2]. Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulation. The tips of the forceps were placed at each side of the paw, and a gradually increasing force was applied. The pressure, in grams, producing withdrawal of the paw or in some rare cases the vocalization of the animal, corresponded to the nociceptive threshold value. This manipulation was performed 3 times for each hindpaw, and the values were averaged. All tests were performed between 10:00 am and 4:00 pm prior to any injection.

2.3. Drugs and treatments

Inflammation was induced by unilateral knee injection of 50 μL CFA (Sigma, St. Louis, MO) following the procedure already published on the tibiotarsal model [8]. The control animals received an equivalent volume of mineral oil, the vehicle of CFA (Sigma). Etifoxine (EFX: Biocodex, Gentilly, France) was prepared in saline (NaCl .9% in distilled water) containing 1% Tween 80 (v/v; Sigma) and injected daily (50 mg/kg intraperitoneally in a final volume of 10 mL/kg). In previous studies, this concentration has been shown to fully alleviate pain symptoms when neuropathic animals received EFX as a prophylactic and/or curative treatment [2,3]. This vehicle solution was used (without EFX) for the control group. To study the effect of etifoxine on pain symptoms, 5 consecutive daily injections were given to rats, 3 days after the CFA injection. Prophylactic EFX treatment started 1 week before the CFA injection and lasted for 3 weeks.

2.4. Quantitative polymerase chain reaction (PCR)

Lumbar spinal cord was collected at day 9, after the 5 injections of EFX (or vehicle) and directly stored at −80°C. Total RNA was extracted according to a protocol consisting of 2 independent total RNA extractions separated by a DNAseI treatment (DNA-free kit, Ambion, Life technologies, Saint Aubin, France) as previously described in detail [24]. RNA quality and concentration were assessed by spectrophotometry and automated electrophoresis on microfluidic chips (Agilent 2100 Bioanalyzer system, Agilent technologies, Les Ulis, France). Total RNA (800 ng/sample) was subjected to reverse transcription using the Iscript kit according to the manufacturer instructions (Bio-Rad, Marnes-la-coquette, France). PCR was set up in 96-well plates using diluted cDNA samples, highly selective primer sets (see sequences in Supplementary material), and SyberGreen-containing PCR reagents (Bio-Rad) accurately dispensed using a robotic workstation (Freedom EVO100 from Tecan, Lyon, France). Gene amplification and expression analyses were performed on a MyIQ real-time PCR machine (Bio-Rad) using a 3-step procedure (15 seconds at 96°C; 10 seconds at 62°C; 15 seconds at 72°C) followed by a melting curve study to ensure specificity of the amplification process. Standardization was made possible using standard curves made from control RNA samples and hypoxanthine phosphoribosyltransferase 1 as housekeeping gene. The differences between samples were calculated on the basis of the specific ratios (gene of interest/housekeeping gene).

2.5. Immunohistochemistry for OX-42

On day 5 of the EFX treatment, rats were perfused intracardially with 150 mL of phosphate buffer (0.1 M, pH 7.4) followed by 500 mL of a solution containing 4% paraformaldehyde .6%, picric acid in phosphate buffer. After laminectomy, the lumbar spinal cord (L2–L5) segments were collected, immersed overnight in the same fixative, and washed the next day in phosphate-buffered saline (PBS). When collected, the lumbar spinal cord was notched on the ventral right side to allow both sides to be distinguished (ipsilateral vs contralateral to the injected knee). Transverse sections of 40 μm were prepared using a tissue slicer (Leica VT1000S, Wetzlar, Germany). The sections were rinsed 3 times during 10 minutes in PBS and subsequently were incubated for 1 hour in a blocking solution composed of 5% donkey serum in PBS and .5% Triton X-100. The sections were then incubated overnight with the primary antibody OX-42 (1:400, Serotec, ref. MCA275R, Raleigh, USA) diluted in PBS containing 1% normal serum and .5% Triton X-100. After a wash with PBS, the sections were incubated for 90 minutes with the secondary biotinylated antibody (1:2000, Santa Cruz Biotechnology Inc., Dallas, USA) and treated with a peroxidase-conjugated avidin-biotin complex (Vectastain Elite ABC Kit, Vector Laboratories) for 1 hour at room temperature. After rinsing with PBS (3 × 10 minutes), ABC reaction was revealed by incubation with 3,3′-diaminobenzidine tetrahydrochloride, .03% H_2O_2 in .05 mL Tris buffer (pH 7.6). The reaction was stopped with distilled water, and the sections were rinsed for 10 minutes with Tris-HCl buffer (pH 7.6) and with PBS (2 × 10 minutes) before being mounted on gela-

tined slides. Finally, the sections were dried overnight and then dehydrated through ascending concentrations of alcohol, followed by xylene. The optical density of the stained lamina I-II was measured on each side of the spinal cord slices (n > 20 per animal) to illustrate ipsilateral/contralateral OX-42 expression ratio in the different experimental groups (n = 6 rats per group).

2.6. BDNF dosage

Lumbar spinal cord was homogenized in extraction buffer (50 mM Tris HCl, 5 mM $MgCl_2$, 1 mM DDT, .5 mM PMSF, .1 mM EDTA, .1 mM EGTA, and .9% NaCl). After a centrifugation step (13,000*g* for 20 minutes), supernatants were collected and stored at −20°C. Prior to BDNF analysis, protein contents were determined using a protein assay kit (Bio-Rad). BDNF proteins were quantified using a rat-BDNF enzyme-linked immunosorbent assay (ELISA) kit for detection (CYT306, Merck-Millipore, Molsheim, France) according to the manufacturer's recommendations. Briefly, 100 μL of the samples diluted (1:2) with the sample diluent provided in the kit were loaded in the 96-well ELISA plate and incubated 12 hours at 4°C. The plates were washed 4 times (300 μL) with wash buffer provided in the kit. Then 100 μL of anti-BDNF antibody (1:1000, v:v) were added in each well and incubated for 3 hours at room temperature. Wells were washed 4 times with wash buffer, and 100 μL of streptavidine HRP (1:1000, v:v) were added and incubated for 1 hour at room temperature. Then, wells were washed 4 times with wash buffer and 100 μL of TBM (solution of 3, 3′, 5, 5′-tetramethylbenzidine in a proprietary buffer with enhancer) were added. Coloration was developed during 15 minutes and blocked by adding 100 μL of stop solution. ELISA plates were read at 450 nm with a plate reader (Thermo Scientific Multi Scan FC, Courtaboeuf, France). BDNF standards were diluted in the solution of sample diluting buffer and extraction buffer (1:1, as for the sample). Standard and samples were loaded in duplicates. The coefficient of variation (CV) values were found between 0% and 7%. All samples with a higher CV value were retested in order to obtain a coefficient of variation $\leqslant$7%. The experimental detection range was estimated between 7.8 pg/mL and 500 pg/mL, a value compatible with the ELISA kit datasheet. Data were expressed as picogram of BDNF per milligram of tissue.

2.7. Prostaglandin E2 assay

Fresh tissue samples were flash-frozen and stored at −80°C. Lipids including eicosanoids were then extracted from tissues in 70% cold ethanol and 30% monobasic sodium phosphate (0.1 M), pH 4.0, followed by shaking incubation for 30 minutes at 24°C. Homogenates were centrifuged at 1000 × g for 10 minutes. Supernatants were evaporated and pellets reconstituted in enzyme immunoassay buffer. PGE2 concentration was determined by ELISA using a PGE2 kit (Caymen Chemical, Talinn, Estonia) according to manufacturer's directions. Experiments were performed in duplicate. PGE2 concentrations were calculated from standard curves using a nonlinear regression equation and expressed in pg/g using a protein assay performed on original pellets, using bovine serum albumin as an internal standard.

2.8. Electrophysiological recordings and data acquisition

Patch-clamp recordings were made from lamina II neurons in transverse slices of mature (>P21) Sprague-Dawley rats following a procedure previously described [23]. Composition of the solution and technical details can be found in the Supplementary materials. All recordings were made at 20°C to 24°C. Whole-cell voltage-clamp recordings were obtained with an Axon MultiClamp 700B amplifier controlled by the PClamp acquisition software (MDS Analytical Technologies, Sunnyvale, CA). Borosilicate glass electrodes with inner filament (1.2 outer diameter to .69 inner diameter; Harvard Apparatus Ltd, Les Ulis, France) were filled with an intracellular solution containing (in mM): 80 Cs_2SO_4, 2 $MgCl_2$, 8 KCl, 10 HEPES, 2 MgATP, .2 NaGTP, 10 Biocytine, pH7.3, CsOH. Series capacitance and resistance were compensated electronically throughout the experiments, and only recordings with series resistance of <40 MΩ were considered for analysis. Recordings were filtered at 5 kHz, digitized at 10 kHz, and stored on a computer before being analyzed. Spontaneous excitatory postsynaptic currents (glutamate) were separated from spontaneous inhibitory postsynaptic currents (sIPSCs; GABA and glycine) by switching the applied holding potential from −60 mV to 0 mV. Synaptic currents were detected offline and analyzed using the Strathclyde electrophysiology software packages winEDR and winWCP (courtesy of Dr. J. Dempster, University of Strathclyde, Glasgow, UK).

In vivo electrophysiology was performed using male Sprague Dawley rats, weighting 300 to 450 g, deeply anesthetized with isoflurane (2% to 3%) pushed by O_2 at a flow rate of about 1 L/min. During the surgery and the recording, the body temperature of the animals was regulated using a temperature-controlled heating blanket (Harvard Apparatus Ltd) maintaining the core temperature of 37°C. The surgical procedure, recording conditions, and analysis have been described in a previously published paper [22] and are provided in the Supplementary materials.

2.9. Statistics

All data are expressed as mean ± standard error of the mean (SEM). One- or 2-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni posthoc test was used to analyze the effects on mechanical sensitivity, whereas Tukey post hoc tests were done on PCR data, PGE2 dosage values, and action potential firing values of spinal neurons in vivo. Other comparisons were made with a 2-tailed Student *t* test for unpaired data. Differences were considered to be statistically significant for $P < .05$.

3. Results

3.1. EFX treatment alleviates the pain symptoms resulting from knee-joint CFA injection

Efficacy of curative and preventive etifoxine treatments on pain symptoms was assessed on rats exhibiting mechanical hyperalgesia of the hindpaw induced by a single unilateral injection of CFA (day 0) in the knee joint (Fig. 1). Mechanical hyperalgesia was seen as a reduction in the mechanical nociceptive threshold sufficient to induce a paw withdrawal reflex and quantified by a calibrated forceps. Hyperalgesia developed rapidly after the CFA injection (1.9-fold reduction in withdrawal threshold compared to baseline) and lasted for at least 3 weeks in our experimental conditions (Fig. 1). For clarity, the figure does not show the time course of pressure thresholds corresponding to (1) paws of animals having received an intra-articular injection of the vehicle of CFA (n = 8), and (2) the contralateral paw of CFA-injected animals (n = 8). These thresholds remained stable around a mean value of about 300 g and were never found to be statistically significant from each other at a given time point or when compared to their respective control (at time 0 before the knee injection). To test for analgesic properties of EFX (Fig. 1A), we started treating animals with a daily injection of EFX (50 mg/kg intraperitoneally) or of its vehicle, 3 days after CFA injection (when hyperalgesia became maximal and stable) and for 5 consecutive days. Note here that pressure thresholds were always measured at least 17 hours after each injection

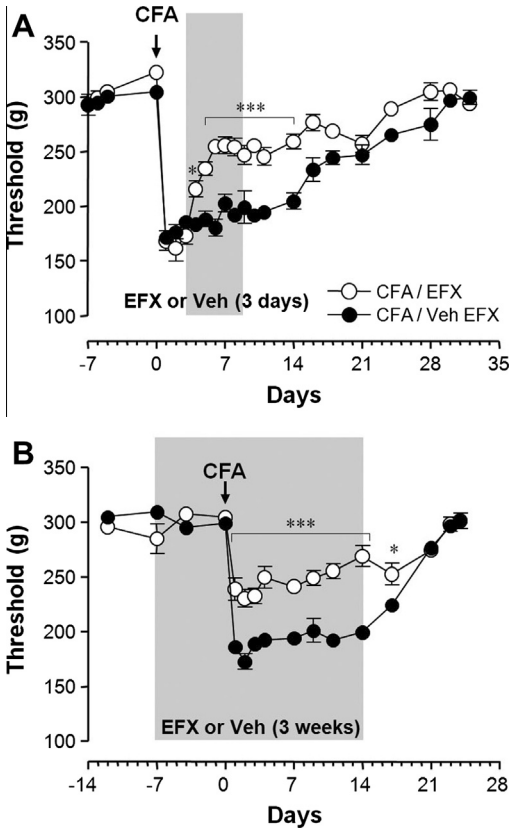

Fig. 1. Effect of prophylactic and curative etifoxine (EFX) treatment on the time course of mechanical hyperalgesia after knee-joint complete Freund's adjuvant (CFA) injection. Both graphs show the time course of the mechanical nociceptive thresholds measured from the ipsilateral paw of animals receiving a curative (A) or a prophylactic (B) treatment of etifoxine. The black arrow indicates the CFA injections in the knee joint made at day 0 after having measured basal thresholds. Treatment with etifoxine (open symbols) or vehicle (filled symbols) lasted 5 days for the curative treatment (A) or 3 weeks for the prophylactic treatment (B). The treatment period is shown in gray. Repeated-measures 2-way analysis of variance showed a significant effect of time ($F_{22,207} = 77.05$, $P < .0001$), EFX treatment ($F_{1,207} = 157.23$, $P < .0001$), and their interaction ($F_{22,207} = 6.81$, $P < .0001$). Asterisks illustrate statistical significance obtained after using Bonferroni post hoc test for each group at a given time ($^{*}P < .05$, $^{***}P < .001$).

(ie, nociceptive tests were performed before the daily injection) in order to characterize long-lasting action of EFX treatment. After the first injection of EFX, the mean pressure threshold was significantly increased compared to the pre-EFX value (D3: 174.9 ± 6.7 g, n = 6; $P < .05$ Bonferroni) and the antihyperalgesic effect was maximal after 3 injections (D6: 254.4 ± 4.2 g; n = 6; $P < .001$ Bonferroni). This corresponded to 60% analgesia in comparison to values obtained from the ipsilateral paw of the vehicle-treated group. When treatment stops, no pain rebound could be observed.

In a second set of experiments, we characterized the prophylactic effect of EFX on the development of mechanical hyperalgesia (Fig. 1B) induced by CFA injection. To do so, EFX (50 mg/kg, intraperitoneal daily injections) was administrated to rats 1 week before intra-articular CFA injection and treatment was maintained for 2 additional weeks (Fig. 1B). No changes in the mean mechanical threshold could be recorded in pain-free animals (ie, before CFA injection) receiving EX or the vehicle (not shown). Pretreatment with EFX did not prevent the appearance of mechanical hyperalgesia following CFA injection, but limited its intensity as indicated by the higher pressure threshold values observed at day 3 (vehicle-treated: 188.9 ± 5.4 g; EFX-treated: 233.2 ± 8.4 g; n = 6; $P < .001$ Bonferroni). With this EFX regimen, intensity of CFA-induced hyperalgesia was reduced by about 50% compared with vehicle-treated animals.

3.2. Etifoxine control of the central inflammatory response

In the nervous system, microglial cells are important players in the inflammatory response. In a first set of experiments, we looked at inflammatory-related events such as activated microglia and production of proinflammatory cytokines. Microglia activation in the lumbar spinal cord was determined through the expression of OX-42 mRNA and protein product, using quantitative reverse transcriptase (RT)-PCR and immunohistochemistry, respectively. We first found a significant overexpression (2-fold increase) of OX-42 transcripts in the ipsilateral spinal cord of CFA-injected animals when compared with the ipsilateral side of control animals (Fig. 2A; CFA vs vehicle-injected: n = 8, $P < .001$, Tukey test). This gene expression induction was fully abrogated by EFX treatment. In good agreement with this observation, immunostaining using OX-42 antibody revealed a slight hyperreactivity in the ipsilateral spinal cord of CFA-treated rats when compared to the contralateral side (Fig. 2B). This difference, expressed as an OX-42 expression ratio between the ipsilateral and contralateral side, was of 1.21 ± .06 (n = 6). After EFX treatment, we failed to see any difference in OX-42 labeling between the ipsilateral and contralateral side as indicated by the OX-42 expression ratio, which was significantly reduced to 1.04 ± .03 (n = 6; $P < .01$, Student t test).

To further examine the spinal inflammatory response after EFX treatment, expression of transcripts encoding for some well-known inflammatory mediators was measured in RNA samples extracted from the lumbar spinal cord of CFA-induced hyperalgesic rats treated from day 3 to day 7 with curative doses of EFX or vehicle (Fig. 2C). Quantitative RT-PCR revealed an important increase of the proinflammatory cytokines TNFα and the interleukins IL-1β and IL-6 that was fully or partially obliterated by EFX (see Table 1 for details). Conversely, expression of the anti-inflammatory cytokine IL-10 was reduced in CFA-injected rats but resumed in EFX-treated animals. Therefore, while CFA triggers change in cytokine production by boosting proinflammatory cytokines and reducing anti-inflammatory ones, EFX treatment seems to counteract CFA effects by resuming cytokine homeostasis. Taken together, the present data suggest that CFA-induced hyperalgesia elicited not only robust ipsilateral microglial activation but also a central inflammatory response associated with a massive expression of proinflammatory cytokines. These inflammatory processes were strongly repressed in animals treated with EFX.

3.3. Etifoxine prevents glycinergic disinhibition and amplifies GABAergic inhibition

The inducible cyclooxygenase type 2 (COX-2), which is responsible for the production of prostaglandins, is a well-known transducer of peripheral inflammatory signals into the nervous system. We looked at COX-2 expression and function using quantitative RT-PCR and PGE2 ELISA assays. As illustrated in Fig. 3A, the expression of COX-2 mRNA was significantly increased by 2.5-fold in the dorsal horn of the spinal cord (n = 6, $P < .001$, Tukey test), ipsilateral to the CFA-injected knee joint, compared to control rats (ie, vehicle knee injection) and by 1.8-fold in the contralateral side of CFA-injected animals (n = 6, $P < .01$, Tukey test), suggesting a rather side-specific upregulation of COX-2 expression in response to unilateral injection of CFA in the knee. Ipsilateral spinal cords collected from EFX-treated CFA rats had restored expression levels of COX-2. In line with the increased expression of COX-2 mRNA levels, ELISA revealed that PGE2 levels were 3 times more elevated in the ipsilateral spinal cord of CFA-injected animals (Fig. 3B) compared to PGE2 levels in the contralateral side (contralateral:

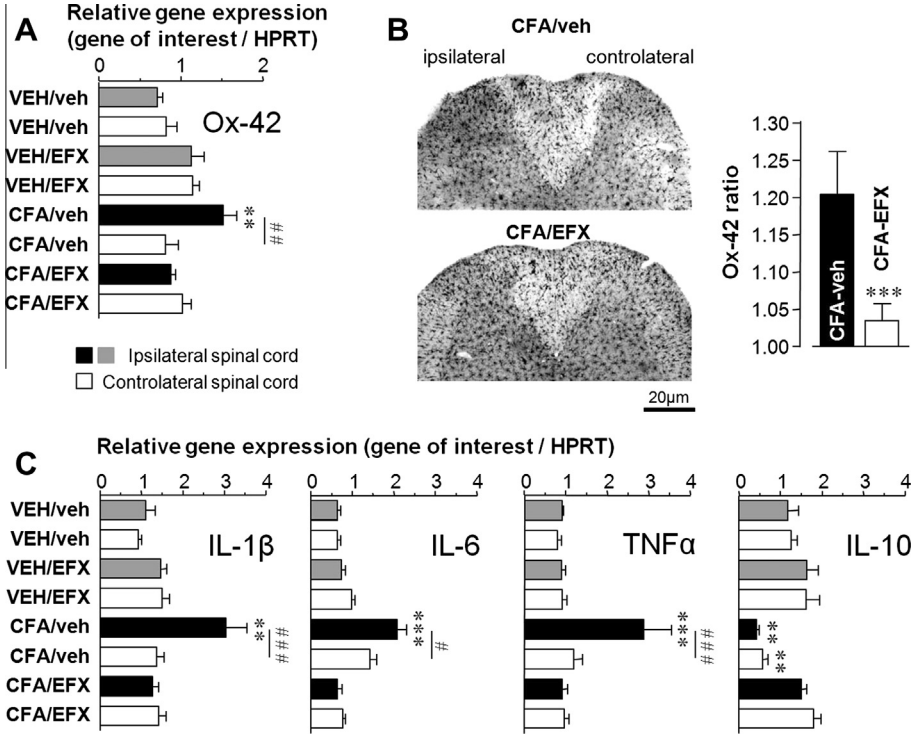

Fig. 2. Effect of etifoxine (EFX) curative treatment on microglial activation and the spinal production of cytokine. (A) Real-time polymerase chain reaction quantification of OX-42 mRNAs in the lumbar spinal cord of rats having received an intra-articular injection of complete Freund's adjuvant (CFA) or of its vehicle (VEH), and then treated with EFX or with its vehicle (veh). Analyses were performed on lumbar spinal cord extracts of 8 male rats per condition. (B) Representative images of the dorsal horn spinal cord after revelation of OX-42 immunostaining in a CFA-injected rat treated with the vehicle of EFX (top) or with EFX (bottom). Expression ratios between the ipsilateral and the contralateral sides are shown on the right histogram for both groups (n = 6). Two-tailed unpaired Student *t* test indicates statistical difference between groups at $P < .01$. (C) Real-time polymerase chain reaction quantification of mRNA coding for the proinflammatory cytokines (interleukin [IL]-1, IL-6, tumor necrosis factor [TNF] α) and anti-inflammatory IL-10. Analyses were performed on lumbar spinal cord extracts of 8 male rats per condition. All data are expressed as mean ± SEM. Symbols indicate statistically significant differences with Bonferroni multiple comparison post hoc tests after 1-way analysis of variance while comparing values from each animal group between side (ipsilateral vs contralateral: $^{\#}$within each group) and comparing ipsilateral values in VEH- and CFA-injected rats treated or not with EFX (veh vs EFX: *between groups). Significance code: $^{*,\ \#}P < .05$, $^{**,\ \#\#}P < .01$, $^{***,\ \#\#\#}P < .001$. HPRT, Hypoxanthine phosphoribosyltransferase.

Table 1
Fold changes in mean expression value for several genes in the SC of VEH and CFA-injected rats (n = 8 per group) treated during 5 days with and without EFX (EFX and veh, respectively).

Gene	Ipsilateral SC		Contralateral SC	
	VEH vs CFA	CFA vs CFA-EFX	VEH vs CFA	CFA vs CFA-EFX
Inflammatory mediators				
IL-1β	**+2.7**	**−2.4**	**+1.5**	+1.0
IL-6	**+3.3**	**−3.3**	**+2.2**	**−1.8**
TNFα	**+3.2**	**−3.2**	−1.2	+1.0
Prostanoid receptors				
EP1	+1.0	−1.0	−1.0	−1.1
EP2	**+1.9**	**−2.1**	−1.0	+1.1
EP3	+1.0	−1.1	−1.1	+1.1
EP4	**+1.7**	**−2.2**	−1.2	+1.0
Glycine receptor subunits				
α1	**−2.3**	**+1.8**	**−1.6**	**+1.4**
α2	−1.3	+1.1	−1.0	−1.0
α3	−1.3	+1.1	−1.3	+1.1
α4	**−1.4**	**+1.4**	−1.3	+1.1

Bold values indicate statistical differences for the selected comparisons at $P < .05$ with Tukey post-hoc tests.
CFA = complete Freund's adjuvant; EFX = etifoxine; IL = interleukin; SC = spinal cord; TNF = tumor necrosis factor; VEH = vehicle-injected rats.

10.0 ± .2 pg/g; ipsilateral: 32.9 ± 2.1 pg/g, n = 8; $P < .001$, Tukey test). PGE2 levels returned to control levels in the spinal cord of CFA-injected rats treated with EFX (ipsilateral: 8.8 ± .7 pg/g of tis-

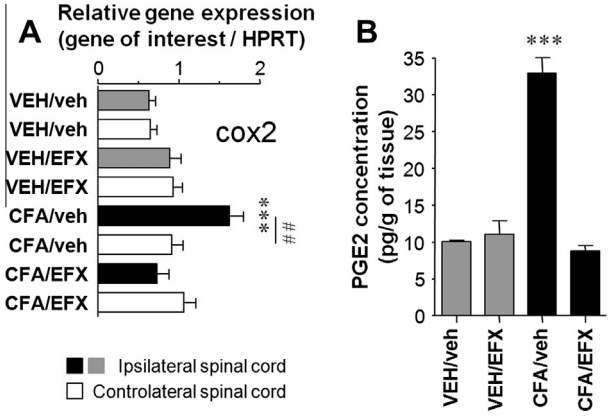

Fig. 3. Expression of cyclooxygenase type 2 (COX-2) mRNA and of one of its enzymatic products, prostaglandin E2 (PGE2), in the lumbar spinal cord of control and complete Freund's adjuvant (CFA)–injected animals. (A) Real-time polymerase chain reaction quantification of COX-2 mRNAs in ipsilateral and contralateral lumbar spinal cord extracts of rats having received a knee injection of CFA or vehicle (VEH), treated or not with etifoxine (EFX) after apparition of mechanical hyperalgesia. (B) Enzyme-linked immunosorbent acid quantification of spinal cord PGE2 for the ipsilateral spinal of the different groups of rat. Data are presented as mean PGE2 content ± SEM. Symbols indicate statistically significant differences with Tukey multiple comparison post hoc tests after 1-way analysis of variance while comparing values from each animal group between sides (ipsilateral vs contralateral: $^{\#}$within each group) and comparing ipsilateral values in VEH- and CFA-injected rats treated or not with EFX (veh vs EFX: *between groups). Significance code: $^{\#\#}P < .01$, $^{***}P < .001$. HPRT, Hypoxanthine phosphoribosyltransferase.

sue, n = 8; $P > .05$, Tukey test). Together, these results strongly support that CFA-induced hyperalgesia is not restricted to the joint but actually generates an ipsilateral-specific proinflammatory response in which elevated PGE2 production driven by inducible cyclooxygenases plays an undoubted role.

COX-2/PGE2–mediated signaling could affect glycinergic inhibition in the spinal cord [1] and be responsible, at least in part, for the appearance of pain symptoms. To test this hypothesis, we analyzed the expression of prostanoid receptors mediating the spinal effects of PGE2. As shown in Table 1, only mRNA encoding for EP2 and EP4 receptors was increased in the ipsilateral spinal cord of CFA-injected animals, whereas EP1 and EP3 remained unaffected. In addition, elevated EP2 and EP4 transcript levels returned to basal threshold after EFX treatment. Because EP2 and EP4 are positively coupled to adenylate cyclase (whereas EP1 and EP3 are negatively), our results suggested that EFX effects on pain and inflammation could modulate EP receptor-mediated phosphorylation of α3-containing glycine receptor channels, leading to spinal disinhibition. Thus, quantitative RT-PCR analysis of specific α subunits of the GlyR was performed in both the ipsilateral and contralateral sides of the spinal cord, isolated from CFA-injected hyperalgesic rats, treated or not with EFX (Fig. 4 and Table 1).

Data revealed that CFA injection resulted in a slight but not significant decrease in the expression of α1 in spinal cord extracts collected from the ipsilateral spinal cord of CFA-injected rats compared to the other conditions (Fig. 4; $P > .05$, Tukey test). Compared to the expression changes of the other α subunits, this led to a significant ($P < .001$, Student t test) difference in the ratio between α1 and α3 subunits (Fig. 4B). To further address the potential physiological relevance of this result, we performed patch-clamp recording of lamina II neurons, known to express α3-containing GlyRs, in order to analyze the functional consequences of EFX treatment on spontaneously occurring GlyR-mediated inhibitory postsynaptic currents (sIPSCs).

Our results revealed that GlyR-induced sIPSCs were significantly smaller in amplitude in CFA-injected rats (Fig. 4C and D). This resulted in a decrease of synaptic inhibitory charge carried by GlyR (Fig. 4D; Table 2: see Area), that was prevented by EFX treatment. We also characterized GABAAR sIPSCs in spinal cord slices of CFA-injected rats (n = 8), treated or not with EFX. As shown in Fig. 5A and B and Table 2, the mean decay time constant and inhibitory current charge (area) of GABAAR sIPSCs was significantly increased in lamina II neurons recorded from EFX-treated CFA rats compared to the vehicle-treated group.

3.4. Etifoxine to protect against chloride-mediated spinal disinhibition

As shown earlier, the prevention of GlyR disinhibition and potentiation of GABAAR function after EFX treatment might easily by explained by its analgesic properties. To further analyze how EFX could protect from spinal disinhibition, we measured mRNA expression for potassium chloride exporters (KCC2) and importers (NKCC1), which control chloride ion homeostasis. Fig. 5C shows transcript expression levels for NKCC1 and KCC2 in the different experimental groups. We observed that NKCC1 expression is only positively affected by CFA from the ipsilateral samples, whereas KCC2 is noticeably reduced in the same condition (Fig. 5C). These changes were, however, not significant between paw side within groups or for the ipsilateral paws of CFA-injected rats between treatments ($P > .05$, Tukey test). These slight antagonistic actions of CFA on potassium chloride transporters led to a more pronounced unbalance of the NKCC1/KCC2 ratio (Fig. 5C). This ratio, which significantly favored chloride importer vs exporter in CFA-injected animals, returned to values seen in control animals when inflamed animals are treated with EFX (not shown; $P < .001$, Student t test). This result might be of interest because a reduction of KCC2 exporter has been shown to be promoted by local release of BDNF leading to excessive intracellular chloride ion concentra-

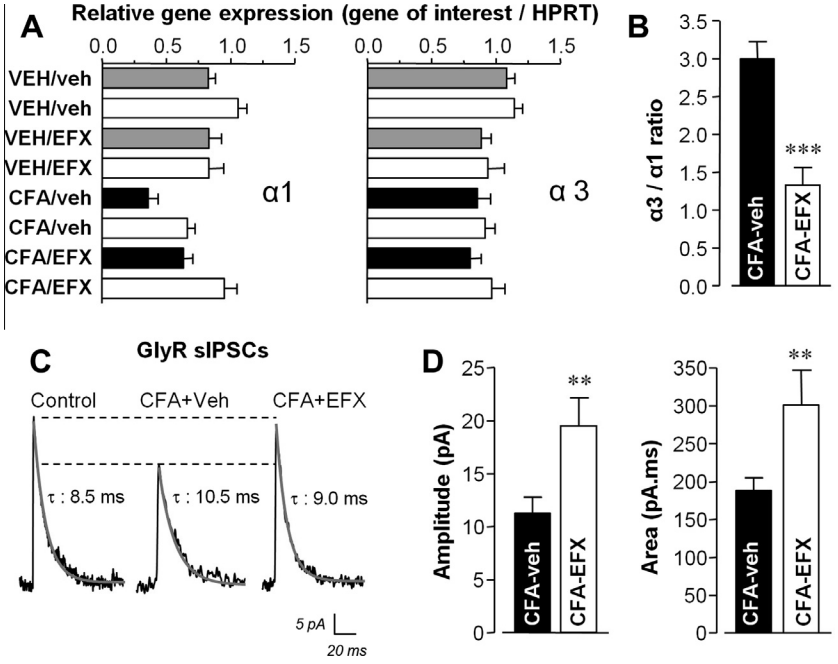

Fig. 4. Etifoxine (EFX) effects on spinal glycinergic control. (A) Expression profile for α1 and α3 subunit mRNA obtained using quantitative polymerase chain reaction assay for the animal group described previously. (B) Histogram illustrating changes in the GlyR α3/α1 mRNA ratio in the spinal cord of complete Freund's adjuvant (CFA)–injected animals. Rats received a curative treatment with EFX (CFA-EFX). Corresponding control subjects received the vehicle (CFA-veh). (C) Representative glycine receptor spontaneous inhibitory postsynaptic currents (sIPSCs) obtained from control rats (vehicle injected), CFA-injected rats not treated (CFA-Veh), and rats treated with EFX (CFA-EFX). (D) Histogram summarizing the effects of EFX treatment on sIPSC amplitude and inhibitory charge (Area) for CFA-injected rats. Asterisks indicate statistical significance at $^{***}P < .001$ and $^{**}P < .01$ using the Student t test on unpaired (polymerase chain reaction ratio) and paired (patch-clamp) data. HPRT, Hypoxanthine phosphoribosyltransferase.

Table 2
Properties of sEPSCs and sIPSCs recorded from lamina II neurons of monoarthritic rats treated with EFX (CFA-EFX, 50 mg/kg intraperitoneally, n = 6) or with its vehicle (CFA-veh, n = 8).

	Area (pA.ms)	Amplitude (pA)	Rise time (ms)	Decay time (ms)	Frequency (Hz)
AMPA-type glutamate sEPSCs					
CFA + veh	−177.2 ± 12.1	−18.3 ± 2.1	1.13 ± 0.11	5.5 ± 0.4	1.90 ± 0.35
CFA + EFX	−168.1 ± 19.9	−20.1 ± 1.7	1.11 ± 0.18	5.9 ± 0.7	1.03 ± 0.34
Glycine sIPSCs					
CFA + veh	188.2 ± 16.8	11.3 ± 1.5	1.08 ± 0.11	8.4 ± 0.5	0.37 ± 0.10
CFA + EFX	**301.8 ± 45.0****	**19.5 ± 2.7****	0.83 ± 0.15	9.6 ± 1.1	0.59 ± 0.23
GABAA sIPSCs					
CFA + veh	278.1 ± 32.8	12.7 ± 1.2	1.11 ± 0.14	20.6 ± 1.5	0.47 ± 0.14
CFA + EFX	**409.1 ± 50.7*****	12.5 ± 1.0	1.05 ± 0.22	**27.5 ± 0.8*****	0.62 ± 0.16

Values in bold indicate statistical differences found between vehicle-treated and EFX-treated animals with the 2-tailed unpaired Student *t* test at $^{**}P < .01$ and $^{***}P < .001$. AMPA = alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CFA = complete Freund's adjuvant; EFX = etifoxine; GABAA = gamma-aminobutyric acid type A; sEPSCs = spontaneous excitatory postsynaptic currents; sIPSCs = spontaneous inhibitory postsynaptic currents; veh = vehicle-injected rats.

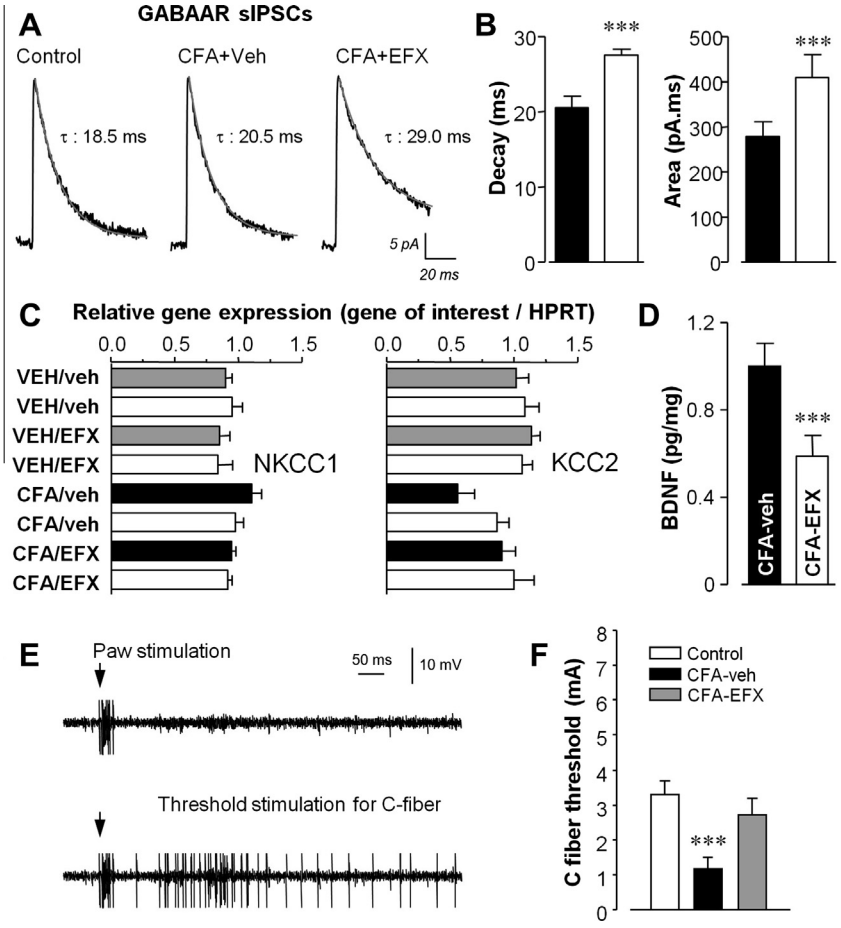

Fig. 5. Effects of a curative treatment with etifoxine (EFX) on spinal inhibition. (A) Representative gamma-aminobutyric acid type A receptors (GABAARs) spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from lamina II neurons of vehicle- and complete Freund's adjuvant (CFA)–injected animals. Note that sIPSCs from EFX-treated CFA animals were massively prolonged. (B) Histogram summarizing the effects of EFX treatment of GABAAR current duration (monoexponential decay time constant) and inhibitory charge (Area). Asterisks indicate significance using 2-tailed unpaired Student *t* test at $P < .001$. (C) Relative expression of mRNA coding for chloride importer NKCC1 (for Na—K—Cl cotransporter type 1) and exporter KCC2 (for K—Cl cotransporter type 2). Ipsilateral paws are indicated by gray and black bars. (D) Histogram showing brain-derived neurotrophic factor (BDNF) concentrations in the spinal cord in CFA-injected animals treated with EFX (white bar) or with its vehicle (black bar). Asterisks indicate significance using 2-tailed unpaired Student *t* test at $P < .001$. (E) Representative action potential discharge of a wide dynamic range (WDR) neuron recorded in lamina V of the spinal cord using extracellular recording in vivo. Stimulation of the receptive field on the paw (arrow) generated several action potentials corresponding to the progressive recruitment of Aβ, Aδ, and C fibers. Bottom trace was obtained by increasing the stimulation intensity and shows additional action potentials that correspond to the activation of slowly conducting C fiber (bigger delay from the stimulus artifact). (F) Histogram showing the mean activation threshold for C fibers while recording WDR neurons from control (vehicle injected) and CFA-injected animals (CFA-veh and CFA-EFX). Asterisks indicate statistical significance at $P < .001$ using Tukey post hoc test after 1-way analysis of variance. HPRT, Hypoxanthine phosphoribosyltransferase.

tions and cell hyperexcitability in various models of pathological pain. In agreement with this finding, and as shown in Fig. 5D, we found that EFX treatment was associated with a reduction in BDNF concentration in the spinal cord (CFA rats treated with vehicle: .97 ± .12 pg/mg of tissue; CFA rats treated with EFX: .60 ± .08 pg/mg of tissue; n = 6, $P < .001$, Student *t* test). Because EFX may protect

against this KCC2-mediated disinhibitory mechanism, we ended up characterizing EFX action on spinal nociceptive processing in vivo.

Fig. 5E illustrates a typical recording made from convergent wide dynamic range (WDR) neurons in lamina V. By stimulating the peripheral receptive field with current pulses of various intensities, we analyzed the resulting unitary action potential discharge. Based on the respective conduction velocities of Aβ, Aδ, and C fibers, this allowed us to determine their activation threshold. In comparison to control values (vehicle injected), we first found that the activation threshold for C fibers was significantly reduced in ipsilateral WDR neurons of CFA-injected rats (control: 3.30 ± .39 mA; CFA-vehicle: 1.17 ± .32 mA, n = 11; $P < .001$, Tukey test). In CFA-injected rats having received EFX for 5 days, C fiber–associated discharge in WDR was only obtained after stimulation of the peripheral receptive fields at 2.72 ± .48 mA (n = 6), a value that was similar to that of control (pain-free) animals (3.38 ± .55 mA, n = 10; $P > .05$, Tukey test). Taken together, these findings likely indicated that EFX treatment restored physiological C fiber activation thresholds and spinal nociceptive processing by WDR neurons. In a subset of experiments, we further determined that 25% of the recorded WDR (n = 5 of 20) were indeed projecting neurons as they exhibited a retrograde action potential after rostral stimulation of the ascending tracts.

4. Discussion

In summary, we show in this study that EFX treatments (curative and/or prophylactic) protect and elevate global inhibitory strength in the dorsal horn of the spinal cord. This general action results from a synergistic action of EFX at several levels: (1) reduction of several spinal inflammatory processes, (2) protection against PGE2-induced glycinergic disinhibition, and (3) potentiation of GABAAR-mediated inhibitory strength. Together, these molecular mechanisms are likely to contribute in large part to the control of spinal nociceptive processing in CFA-induced pain states (as shown by our in vivo electrophysiological data). These findings are also consistent with a reduction in mechanical hyperalgesia.

Numerous reports indicate that peripheral inflammation resulting from tissue injury (including neuropathic insults) is accompanied by the activation of glial cells in the central nervous system [36]. In the spinal cord, early activation of microglia is particularly well observed in the dorsal horn, in layers in charge of processing peripheral nociceptive messages in experimental monoarthritis [34]. After inflammation or injury, microglial cells become less ramified, begin to proliferate, and while activated, start releasing pronociceptive substances that enhance pain responses [26,36,50]. As shown in several studies on experimental monoarthritis, this includes the release of numerous proinflammatory mediators in the spinal cord [4,5,7,14,43,45,47,49]. In agreement with these findings, we also found significant elevation of mRNA coding proinflammatory cytokines (IL-1β, IL-6, TNFα), PGE2 synthesizing enzyme COX-2 and of PGE2 itself (Figs. 2 and 3). Overexpression of these mRNA was correlated with a significant elevation of OX-42, a classic marker of activated microglia that encodes the integrin alpha M chain. The lateral selectivity of these changes strongly supports the idea that they may result from nociceptive-driven stimulation of the ipsilateral dorsal root ganglion (DRG) and spinal structures rather than a systemic and possibly supraspinal process recruited by the local inflammation in the knee joint.

Early activation of microglia, followed by activation of astrocytes, is thought to contribute in large part to the central sensitization and to the persistence of pain symptoms in various models of pain [36], including monoarthritic models [33,45,47]. In our study, we have been able to massively reduce mechanical hyperalgesia (>60%) with etifoxine (50 mg/kg), and this effect was associated not only with a reduction of OX-42 (mRNA expression and protein immunoreactivity) but also with a reduction of several proinflammatory cytokines, known to contribute to hypernociception. Interestingly, COX-2 mRNA levels and PGE2 concentrations also were reduced by EFX treatment, likely suggesting that part (if not all) of its action was mediated by the control of spinal inflammatory processes. In line with this observation, EFX treatment recently was shown to reduce the number of infiltrated macrophages in cryolesioned sciatic nerve [17]. This novel mechanism of EFX is consistent with some other studies that demonstrate that controlling microglia activation and the subsequent spinal inflammatory response after knee joint inflammation is an efficient way to reduce pain symptoms [33,45,47]. In all cases, the mechanisms responsible for the specific effect of EFX are still unknown and will require further investigation This is particularly the case for the possible functional interaction between EFX and COX activity, which could be of significant interest if demonstrated. An alternative hypothesis is related to the synthesis of steroids, some of them being capable of demonstrating anti-inflammatory properties. For example, EFX binding on mitochondrial TSPO may promote the synthesis of progesterone and of progesterone-derived corticosteroids by (peripheral and central) nerve cells to achieve its anti-inflammatory effect, as previously shown [10,11,20,25].

Apart from the consequence of EFX treatment on gene expression, which likely involves (direct or indirect) genomic action, we also show that inhibition of synaptic transmission mediated by spinal glycine and GABAA receptor channel is protected and amplified, respectively. Using a combined approach with electrophysiological and molecular biology tools, our experimental results strongly suggest that EFX treatment may be responsible for the maintenance of optimal chloride inhibition. Chloride gradients that are disrupted in some pain states, due to the downregulation of KCC2 exporters [12,13], are unlikely to be affected after EFX treatment. Indeed, we found that CFA-injected animals had reduced levels of KCC2 mRNAs in the ipsilateral spinal cord, and this was not the case for the corresponding animals with pain that were treated with EFX. This fits well with the reduced levels of BDNF, which were shown to be at least produced by microglia and responsible for the decrease of KCC2 expression [12]. Interestingly, in models of CFA-induced inflammation, the increase of BDNF mRNAs and protein levels is preeminent in the ipsilateral side [15]. This fully fits with our result,which suggests that EFX analgesia is attributed to a reduction of BDNF release. It is important to keep in mind that BDNF might be released by microglia, but also by a subset of DRG neurons [40]. After its synthesis, BDNF is anterogradely transported to the dorsal horn of the spinal cord, where it has been shown to exert a proinflammatory role, as previously described [27]. Due to our treatment regimen, we cannot exclude a peripheral effect of EFX on DRG neurons in vivo at this stage. Similar to previous findings [1], we found that increased levels of PGE2 were associated with a reduced glycinergic synaptic inhibition in spinal cord neurons. Again, EFX-treated animals had normal levels of PGE2 and GlyR synaptic currents. So far, we have no direct evidence in the literature indicating that EFX targets GABAARs to exert its analgesic effect. Here, we show that EFX-treated animals exhibit prolonged GABAAR synaptic currents, thus strongly demonstrating that potentiation of GABAergic inhibition contributes to the limitation of spinal nociceptive processing. This conclusion is further supported by our in vivo unitary recording of wide dynamic range neurons (some of them being projecting neurons), which are less excitable and display reduced threshold values of C fiber–related action potential discharges.

In summary, we used a multidisciplinary approach to dissect the molecular mechanisms by which EFX may induce its effects on behavioral, electrophysiological, and cellular parameters

dysregulated in CFA-induced monoarthritic pain. For the first time, we demonstrate that EFX antihyperalgesia is mediated by the optimization of chloride inhibition, at least in the spinal cord. This is achieved by the maintenance of proper expression levels for KCC2 exporters, prevention of PGE2-induced glycinergic disinhibition, and amplification of synaptic GABAAR tone. In addition, novel mechanisms of EFX are demonstrated on the inflammatory reactivity in the spinal cord of monoarthritic animals. Indeed, EFX treatment is associated with a reduced activation of spinal microglia, reduced mRNA levels for proinflammatory cytokines, and low concentrations of PGE2 and BDNF. Taken together, the potent action of EFX on generalized neuropathic pain symptoms [2] and on knee-joint inflammatory pain symptoms (this study) is likely explained by these pleiotropic effects, all considered in the current literature as major contributors in the development and maintenance of persistent pain states.

Conflict of interest statement

The other authors have no conflict of interest.

Acknowledgments

This work was supported by the following French institutions: Centre National de la Recherche Scientifique, Université de Strasbourg, and the Institut Universitaire de France (P.P., P.E.J.). M.A. was a PhD fellow from the French Ministère de la Recherche et de l'Enseignement Supérieur. P.P. received funding from Biocodex Laboratories.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pain.2013.11.003.

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