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Characterization of the fast GABAergic inhibitory action of etifoxine during spinal nociceptive processing in male rats

P.E. Juif, M. Melchior, P. Poisbeau*

Centre National de la Recherche Scientifique and University of Strasbourg, Institute of Cellular and Integrative Neurosciences, Strasbourg, France

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ABSTRACT

Etifoxine (EFX) is a non-benzodiazepine anxiolytic which potentiate $GABA_A$ receptor ($GABA_AR$) function directly or indirectly via the production of 3α-reduced neurosteroids. The later effect is now recognized to account for the long-term reduction of pain symptoms in various neuropathic and inflammatory pain models. In the present study, we characterized the acute antinociceptive properties of EFX during spinal pain processing in naive and monoarthritic rats using in vivo electrophysiology. The topical application of EFX on lumbar spinal cord segment, at concentrations higher than 30 μM, reduced the excitability of wide dynamic range neurons receiving non-nociceptive and nociceptive inputs. Windup discharge resulting from the repetitive stimulation of the peripheral receptive field, and recognized as a short-term plastic process seen in central nociceptive sensitization, was significantly inhibited by EFX at these concentrations. In good agreement, mechanical nociceptive thresholds were also significantly increased following an acute intrathecal injection of EFX. The acute modulatory properties of EFX on spinal pain processing were never seen in the simultaneous presence of bicuculline. This result further confirmed EFX antinociception to result from the potentiation of spinal $GABA_A$ receptor function.

1. Introduction

Several mechanisms of spinal disinhibition associated with pathological pain states have recently been characterized and potentiation of GABAergic inhibitory controls in the spinal cord is thought to be an efficient strategy to limit or prevent pain symptoms (Zeilhofer et al., 2012b). Indeed, intrathecal injections of positive allosteric modulators of $GABA_A$ receptors ($GABA_ARs$) function, such as benzodiazepines or 3α-reduced neurosteroids, reduced pain responses in various animal models and in human pain states (Goodchild and Serrao, 1987; Serrao et al., 1992). Results from these studies have, however, been difficult to interpret because of sedative, anxiolytic, and rewarding properties of these compounds. To overcome this difficulty, a growing number of studies are now attempting to use subtype-selective benzodiazepines (Zeilhofer et al., 2012a). In the case of neurosteroids, we recently adopted an alternative strategy aimed at stimulating their endogenous production with translocator protein (TSPO) agonists (Rupprecht et al., 2010). This strategy already appeared to be efficient in some models of pain while using olesoxime (TRO19622), a cholesterol derivative with neuroprotective properties (Bordet et al., 2008), for example. In our laboratory, we demonstrated that 3α-reduced neurosteroids produced after TSPO stimulation with the benzoxazine etifoxine (EFX) were responsible for the long-lasting analgesic effects, seen in several animal models of neuropathic and inflammatory pain (Aouad et al., 2009, 2014a, 2014b). Analgesic mechanisms included amplification of $GABA_AR$-mediated transmission, protection from prostaglandin E2-induced glycinergic disinhibition, reduction of pro-inflammatory processes and maintenance of proper chloride gradients (Aouad et al., 2014b).

Apart from these long-term effects mediated by TSPO, little is known on the acute modulation of $GABA_AR$ function by EFX in the spinal cord and its impact in spinal pain processing. Etifoxine (EFX) is commercially-available as a non-benzodiazepine anxiolytic in several countries (Micallef et al., 2001; Nguyen et al., 2006; Servant et al., 1998) and exerts positive allosteric modulation of β2/β3-containing $GABA_ARs$ (Hamon et al., 2003; Schlichter et al., 2000; Verleye et al., 1999, 2001). These subunits are likely to constitute most of the $GABA_ARs$ since they are widely expressed in all laminas of the spinal cord in rodents (Bohlhalter et al., 1996; Paul et al., 2012) and human (Waldvogel et al., 2010). So far, the precise

* Corresponding author. CNRS, UPR-3212, Département Nociception et Douleur, 21 rue René Descartes, 67084, Strasbourg, France. Tel.: +33 3 68 85 14 76; fax: +33 3 88 61 33 47.
E-mail address: poisbeau@inci-cnrs.unistra.fr (P. Poisbeau).

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location of EFX binding site on β subunit is not known. It is, however, apparently not overlapping with specific sites for benzodiazepine and neurosteroids since the potentiating action of EFX is not altered after binding of the silent benzodiazepine site antagonist flumazenil or of the neurosteroid allopregnanolone (Verleye et al., 1999, 2001). Using freshly dissociated spinal cord neurons, potentiation of $GABA_AR$ currents was observed with low micromolar concentrations of EFX (Schlichter et al., 2000).

To characterize the acute action of EFX on spinal nociceptive processing, we recorded deep dorsal horn neurons (i.e. wide dynamic range neurons), integrating peripheral noxious and non-noxious informations, in anesthetized adult rats. Action potential (AP) discharges resulting from the activation of non-noxious and noxious sensory neurons were analyzed in protocols of acute nociceptive stimulation of the receptive field (RF) and during short-term potentiation of action potential discharge (windup) induced by repetitive stimulation, as previously published (Juif and Poisbeau, 2013). Complementary experiments on a model of knee monoarthritic have also been performed.

2. Material and methods

Male Sprague Dawley rats (250–350 g; Janvier, Le Genest St Isle, France) were used for this study. They were housed by group of 4 under standard conditions (room temperature [22 °C], 12–12 h light–dark cycle) with *ad libitum* access to food and water. All experiments were conducted in conformity with the recommendations of the European Union directive on animal experimentation (2010/63/EU adopted on September 22, 2010) and were evaluated by the regional ethic committee in charge of animal experimentation (CREMEAS, authorization AL 01/01/02/11). This study was conducted under the responsibility of authorized personnel (license 67-116 from the French Department of Agriculture to PP).

2.1. In vivo electrophysiology

Single unit extracellular recordings were made from dorsal horn neurons in the lumbar enlargement of the spinal cord of the rat following the procedure previously described elsewhere (Juif and Poisbeau, 2013). Briefly, a laminectomy was performed in anesthetized rats (isoflurane; Vaporizer Isotec 3 datex-Ohmeda) to expose the L4–L5 segments of the spinal cord. Before recordings, the cord was firmly attached by vertebral clamps and meninges were delicately removed, and the spinal cord surface was covered with a thin layer of mineral oil. Single-unit extracellular recordings were made with a stainless steel electrode (FK#02; FHC, UK) connected to a differential amplifier (DAM80, WPI). An electrode was lowered into the dorsal horn to record neurons located in the deep dorsal horn of the spinal cord. Data were acquired and analyzed by a CED 1401 analog-to-digital interface coupled to a computer with Spike 2 software (Cambridge Electronic Design, Cambridge, UK). All neurons included in the present study were wide dynamic range (WDR) neurons (Le Bars and Cadden, 2009) responding to both innocuous and noxious stimuli after electrical stimulation of the peripheral hind paw RF and located in the medial part of the deep layers of the dorsal horn (752 ± 27 μm; n = 12). Note that 25% of them were found to project to supraspinal structures (Juif and Poisbeau, 2013). After stimulation of the RF, the recorded neuron emitted APs. Two protocols of stimulation were done: (i) 60 stimulations at a frequency of 0.2 Hz (*i.e.* 5 min of recording); stimulus intensity of 1.5 times C-fiber threshold; pulse duration of 1 ms and (ii) wind up (30 stimulations, frequency: 1 Hz, intensity: 3xC-fiber threshold, pulse duration: 1 ms). Wind up efficiency was assessed through the slope and was calculated as a ratio (number of action potentials emitted by the neuron after last stimulation and divided by the number of APs triggered by the first stimulation).

Post-stimulus histograms were built by counting the number of APs corresponding to the activation of fast-conducting Aβ (delay to stimulus artifact <20 ms), slow-conducting Aδ (delay of 20–90 ms) and very slow-conducting C fibers (delay of 90–300 ms), as it is described in the literature for rats weighting about 250 g or more and used for electrophysiology experiments (Urch and Dickenson, 2003). With such animals, APs observed 300–800 ms after the stimulus artifact were considered as being part of the postdischarge. For this experimental approach, it is assumed that most of the non-nociceptive and nociceptive informations are mostly transmitted via Aβ fibers and C fibers, respectively in naïve animals. AP changes were compared before and immediately after the topical EFX application on the spinal cord.

2.2. Behavioral testing

All animals were habituated to the room and to the tests at least one week before starting the experiments. Mechanical nociceptive thresholds were measured using a calibrated forceps (Bioseb, Vitrolles, France) as previously (Aouad et al., 2009). Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps are placed at each side of the paw and a gradually increasing force was applied. The pressure, in gram, producing withdrawal of the paw or in some cases the vocalization of the animal, corresponds to the nociceptive threshold value. This manipulation was performed three times for each hindpaw and the values were averaged.

2.3. Drugs and treatments

EFX (Biocodex, Gentilly, France) was prepared in saline (NaCl 0.9% in distilled water) containing 1% tween 80 (v/v; Sigma, St Louis, USA) and was injected intrathecally 20 min before behavioral testing (dose: 0.6 μg in 20 μl). EFX was applied at the surface of the spinal cord during in vivo electrophysiological recording at 3 different concentrations: 5, 30 and 60 μM. Bicuculline (Sigma–Aldrich, France) was diluted in saline and administered on the spinal cord at a final steady-state concentration of 10 μM. Analysis was performed 15 min after EFX application. After this period, EFX was washed out from the saline solution covering the exposed spinal cord segment.

At the end of the study, EFX was also tested using in vivo electrophysiology of WDR neurons on monoarthritic rats, one week after a unilateral knee injection of 50 μl CFA (complete Freund's adjuvant; Sigma St Louis, MO, USA). The control animals received an equivalent volume of mineral oil, the vehicle of CFA, as previously published (Aouad et al., 2014b).

2.4. Statistics

All data are expressed as mean ± standard error of the mean (SEM). Repeated measure one-way ANOVA followed by Bonferroni comparisons was used to analyze the effects on AP firing while recording from spinal neurons in vivo. Student's t test helped compare the electrical thresholds between two groups (unpaired) or before/after drug application (paired). When parametric tests were inappropriate (low N number of observations or data not normally distributed, A Kruskal–Wallis test or Wilcoxon matched pair test was used. In this later case, Dunn's multiple comparisons posthoc test was used to compare the experimental value to the control. Differences were considered to be statistically significant for $p < 0.05$.

3. Results

3.1. Etifoxine increases mechanical nociceptive threshold and reduces the excitability of WDR neurons after peripheral nociceptive stimulation

In freely-moving animals, mean mechanical thresholds were of 583.3 ± 19.7 g (n = 7) and they remained unchanged after injection of the vehicle of EFX (Fig. 1B; 542.4 ± 14.3 g; n = 5). In sharp contrast, EFX injection resulted in a significant increase of mechanical threshold, which reached a mean value of 768.1 ± 22.2 g (n = 5; Wilcoxon, $p < 0.05$). Compared to control, this corresponded to an increase by about 42% thus confirming the acute anti-nociceptive properties of EFX when spinally administered.

To further analyze EFX action on spinal nociceptive processing, we recorded WDR neurons and first characterized its possible effect of the electrical activation threshold required to observe an A and C-mediated action potential (AP) discharge in WDR neurons (representative traces in Fig. 1A). Compared to control (i.e. before application), we failed to see any changes in the thresholds while using concentration of 5 μM (Fig. 1C). When compared to control (basal in Fig. 1C), this increase was particularly robust after application of EFX at 30 μM (Aβ: from 4.3 ± 0.3 V to 6.3 ± 0.5 V, n = 8; one-way Anova, $F_{4,40} = 17.83$, Bonferroni $p < 0.01$; C: from 21.7 ± 0.4 V to 26.4 ± 0.3 V, n = 12; Bonferroni $p < 0.001$) and at 60 μM (Aβ: 7.4 ± 0.3 V, Bonferroni $p < 0.001$, Aδ: from 15.0 ± 1.2 V to 23.2 ± 0.6 V, n = 5; Kruskal Wallis, $p < 0.01$, Dunn's comparisons $p < 0.05$ and C: 32.1 ± 0.6 V; Bonferroni $p < 0.001$). This increase was fully abolished when EFX (60 μM) was co-administered with bicuculline (10 μM) confirming that this change was mediated by $GABA_A$ receptors.

EFX effects on spinal nociceptive processing were next characterized by quantifying changes in the number of APs emitted by WDR with respect to the respective contribution of Aβ, Aδ and C sensory neurons. Using a stimulation intensity of 1.5 times the threshold for C fibers (frequency: 0.2 Hz; pulse duration: 1 ms), we only observed a significant decrease in the number of APs after

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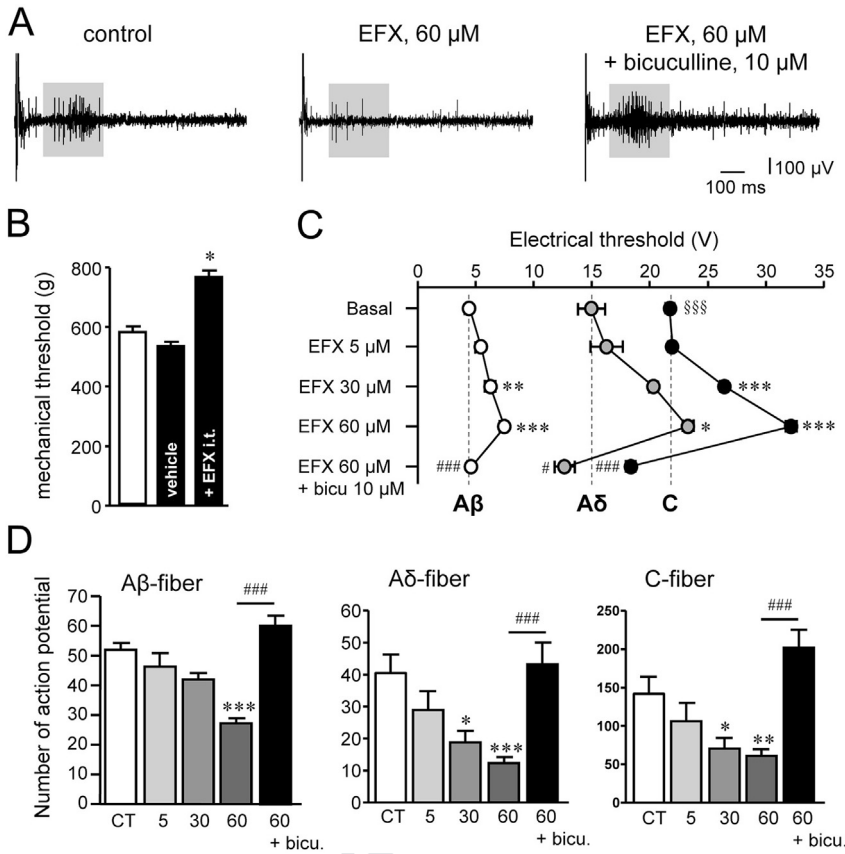

Fig. 1. A. Typical traces representing a single electrical stimulation (intensity: 3 times C-fiber threshold) under basal conditions (left) and following etifoxine (EFX, 60 µM; middle) or EFX (60 µM) + bicuculline (10 µM; right). B. Mechanical threshold (in gram) before (white bar, n = 7) and following (black bar) intrathecal injection of EFX (0.6 µg/20 µl, n = 5) or its vehicle (n = 5). C. Electrical threshold of Aβ- (white, n = 12), Aδ- (grey, n = 4) and C-type (black, n = 12) fiber measured before and following EFX application on the spinal cord. D. Histograms representing the dose response effects of EFX on Aβ (left, n = 12), Aδ (middle, n = 12) and C (right, n = 12) fiber-related AP discharge. Compared to control (CT, white bars), EFX effects are indicated at 5, 30 and 60 µM. Black bar on the right represent the application of EFX at 60 µM in the presence of bicuculline (bicu.).Statistics: Wilcoxon matched pairs test (panel B) and ANOVA followed by Bonferroni comparisons (control vs. treatment): (*)$p < 0.05$, (**)$p < 0.01$, (***)$p < 0.001$; In panel C: (§§§) $p < 0.001$ while comparing Aβ vs C-fiber threshold; (###) $p < 0.001$: EFX vs EFX + bicuculline and (*): control vs treatment).

application of EFX at 30 µM and 60 µM (Fig. 1D). At the maximal concentration tested of 60 µM, EFX significantly reduced the number of Aβ-mediated APs (from 51.9 ± 2.8 to 27.2 ± 1.7, n = 12; one-way Anova, $F_{4,40} = 14.5$; $p < 0.001$; Bonferroni $p < 0.001$), Aδ- (from 40.6 ± 5.9 to 12.4 ± 1.9, n = 12; one-way Anova, $F_{4,40} = 8.0$; $p < 0.001$; Bonferroni $p < 0.001$) and C-mediated fibers (from 142.2 ± 21.9 to 61.2 ± 8.5, n = 12; one-way Anova, $F_{4,40} = 8.4$; $p < 0.01$; Bonferroni $p < 0.01$). Note that APs, mediated by Aδ- and C-, but not Aβ fibers were already significantly reduced with EFX at 30 µM. We also observed that reduction in the number of AP by EFX was not seen when bicuculline was co-applied at 10 µM (number of APs emitted by C-type fibers: control: 142.2 ± 21.9 EFX + bicuculline: 202.1 ± 23.1, n = 6; Bonferroni $p > 0.05$).

3.2. Intrathecal EFX limits short-term potentiation of action potential discharge through a $GABA_A$R-mediated action

Short-term potentiation of AP discharge (windup) has been triggered here using a train of repetitive stimulation of the peripheral RF at constant intensity (3 times the C-fiber threshold, pulse duration: 1ms) and frequency (1 Hz). With the selection procedure used to record WDR neurons (i.e. displaying A and C discharges), we found that all neurons exhibited windup with our stimulation procedure (see representatives traces in Fig. 2A). Windup remained unaffected by a concentration of 5 µM EFX (not shown) whereas it was strongly limited at 30 µM and above (n = 8). This effect can be quantified with the windup ratio (see methods, Fig. 2B). It was significantly reduced in the presence of EFX at 30 µM (ratio = 11.0 ± 1.6, n = 8; one-way Anova, $F_{4,40}$: 48.91; $p < 0.001$; Bonferroni $p < 0.001$) and at 60 µM (ratio = 11.3 ± 1.5, n = 12; one-way Anova, $F_{4,40}$: 48.91; $p < 0.001$; Bonferroni $p < 0.001$), compared to the control windup ratio (17.4 ± 3.1, n = 12). When EFX was co-administered with bicuculline, the windup ratio was similar to the control (15.2 ± 1.7; Bonferroni $p > 0.05$). EFX-induced reduction in the number of APs during windup could also be seen as a reduction in the windup slope before reaching the plateau phase as illustrated in Fig. 2C–D. Note that the reduction in windup ratio and slope was absent when EFX (60 µM) was co-applied with the $GABA_A$R antagonist bicuculline (10 µM). All effects were transient (seen after 15 min) and rapidly washed out (30 min) after removal of EFX.

3.3. EFX action in the monoarthritic pain model

To go one step further, EFX action was characterized in a rat model of persistent inflammatory pain. Knee monoarthritis was induced by an intra-articular injection of complete Freund's adjuvant giving rise to a local inflammation and the development of mechanical and thermal hot hyperalgesia (Aouad et al., 2014b). While recording from WDR neurons in anesthetized CFA-injected rats, we first confirmed this nociceptive hypersensitivity since electrical threshold to observe C-related action potentials was very low (15.1 ± 1.8 V, n = 10) compared to the control groups (oil

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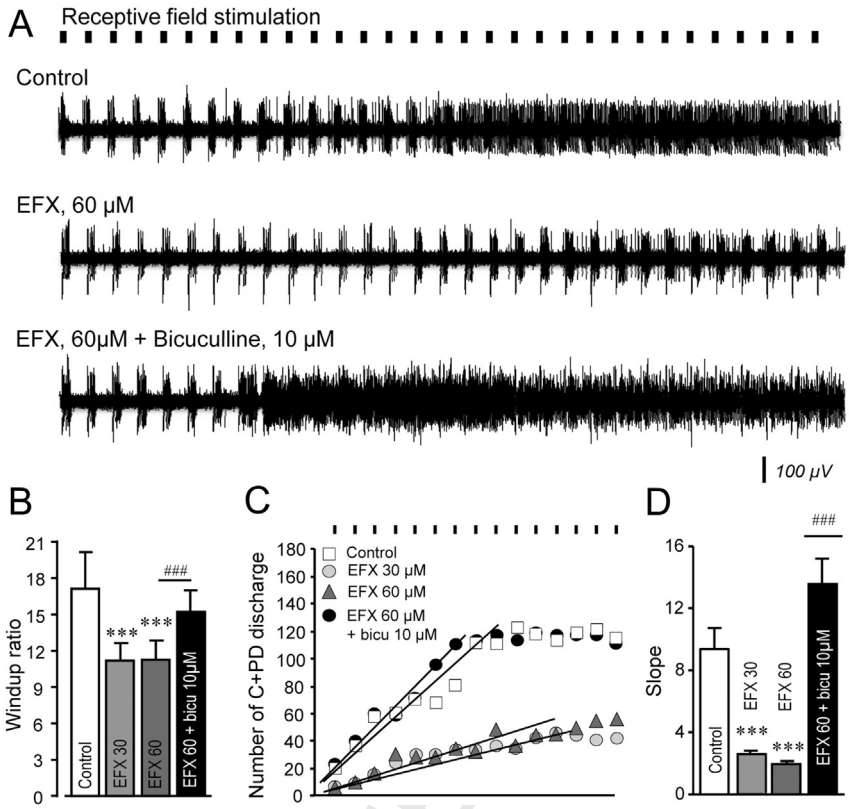

Fig. 2. A: Representative traces illustrating single unit wide dynamic range neuron displaying windup (30 stimulations, 3 times the C-fiber threshold, frequency: 1 Hz) before (CT, top trace) and after (middle trace) EFX (60 μM). The bottom trace represents the responses when EFX (60 μM) is co-applied with the $GABA_AR$ antagonist bicuculline (10 μM). In order to clarify the figure, all the artifacts of stimulation have been removed. B: Histogram showing the windup ratio for the different experimental conditions: control conditions (white bars, n = 12); after EFX application at 30 μM (light grey, n = 8) and at 60 μM (dark grey, n = 12). Black bar shows the windup ratio after application of EFX at 60 μM in the simultaneous presence of the $GABA_A$ receptor antagonist (at 10 μM). C, D: Evolution of the initial slope for a representative WDR neuron (graph in C) and mean slope values for all recorded neurons (graph in D, same N number than in panel B). Statistical code: (*** control vs. EFX treatment; ###, EFX 60 μM vs. EFX 60 μM + Bicuculline 10 μM) significativity at $p < 0.001$ with Bonferroni comparisons after repeated measures one-way ANOVA.

injected: 21.7 ± 0.5 V, n = 8; Student's t = 3.443; df = 10.31; $p < 0.01$). Hyperexcitability of the neighboring neurons and of the recording WDR cell after electrical stimulation did not allowed us to accurately measure threshold for A fibers and forced us to focus our attention on C fiber threshold. After application of EFX at 60 μM, we did observe a significant increase in C fiber threshold which reached 20.2 ± 2.3 V (n = 10, Student's: t = 4.594, df = 9, $p < 0.01$). As illustrated in Fig. 3, EFX effect on C fiber threshold (panel A) was not accompanied by a change in windup ratio (panel B–C, n = 7, Students, t = 1.427, df = 6, $p > 0.05$).

4. Discussion

In this study, we show that the fast non-genomic antinoceptive effect of EFX is mediated by a $GABA_AR$-mediated inhibition in the dorsal horn of the spinal cord. This inhibition is associated with an increase in the mean mechanical nociceptive threshold in freely-moving naïve animals, an overall increase in the electrical threshold to observe A- and C-mediated discharges in WDR neurons and a limitation of windup during repetitive RF stimulation. In monoarthritic rats, EFX antinociception was seen as an increase in C fiber threshold since windup appeared unchanged.

This fast inhibitory action of EFX on spinal nociceptive processing, seen about 15 min after the in vivo application, is consistent with a potentiation of $GABA_AR$ function (Hamon et al., 2003; Schlichter et al., 2000; Verleye et al., 1999, 2001). This potentiation may occur at different places in the dorsal horn of the spinal cord because EFX-sensitive $GABA_ARs$, containing β2-3 subunits, are theoretically expressed by primary afferents of sensory neurons and by most, if not all, dorsal horn neurons (Bohlhalter et al., 1996; Waldvogel et al., 2010). Here, we observed a rather non-specific inhibition of the AP discharge resulting from the activation of C- and A-fibers. Without excluding other possibilities, this likely suggests an elevated $GABA_AR$ inhibitory control onto the recorded WDR neurons limiting their general excitability after a noxious stimulation of the peripheral RF. Another alternative deals with a non-specific increase in the presynaptic inhibition of primary afferents. In any case, an increased efficacy of GABAergic interneurons is involved and perfectly explains the observed EFX effects. Indeed, intrathecal injections of $GABA_AR$ agonists and of allosteric-positive $GABA_AR$ modulators have previously proven their efficacy to reduce pain thresholds in human and animal models (Clavier et al., 1992; Eaton et al., 1999; Edwards et al., 1990; Goodchild and Serrao, 1987; Knabl et al., 2008; Serrao et al., 1992). On the other hand, GABAergic transmission is essential to prevent the appearance of pain symptoms as for example observed after intrathecal injections of bicuculline in vivo (Charlet et al., 2008; Ishikawa et al., 2000; Sivilotti and Woolf, 1994). Our results on monoarthritic rats revealed that EFX antinociception is mediated by an increase in the activation threshold of C fibers and apparently no longer by an inhibition of windup. This interesting result may suggest that presynaptic inhibition of C-type primary afferent fibers by EFX, presumably by potentiating $GABA_AR$ function, is preserved in inflammatory pain states whereas windup inhibition

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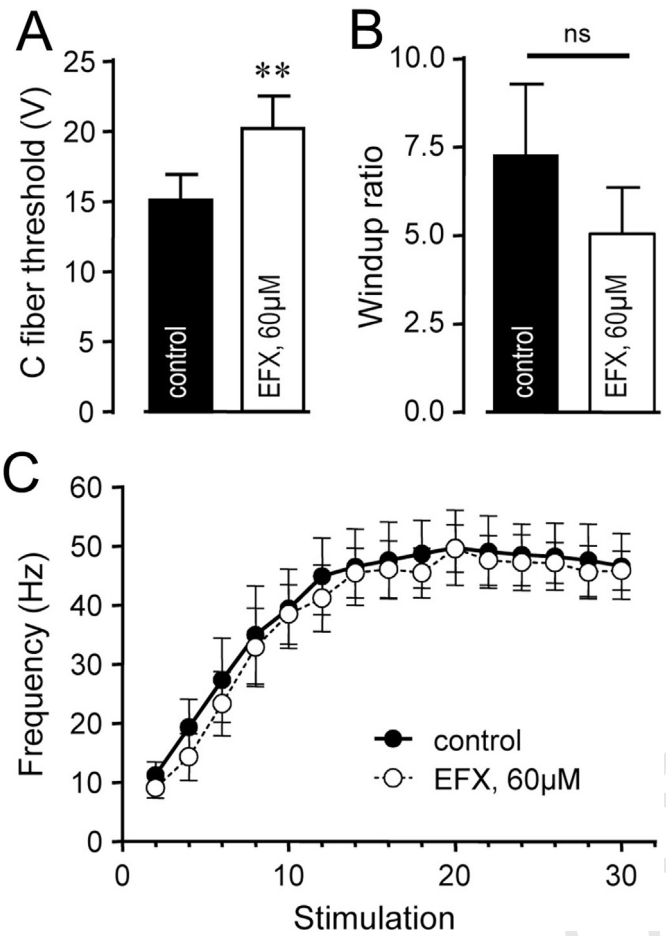

Fig. 3. Etifoxine effects in monoarthritic rats. A: Histogram illustrating C fiber threshold before and 15 min after local spinal application of EFX (60 µM) B,C: Windup ratio (B) and development (C) before and 15 min after EFX application at 60 µM. Statistical code: (**), $p < 0.01$ Student's t-test, control vs. EFX).

exhibited by WDR neurons is no longer possible. This result can be fully explained by a preserved presynaptic shunting effect of $GABA_A$Rs on the primary afferent C-fibers combined to spinal disinhibition of dorsal horn layer neurons (like WDR neurons) processing nociceptive messages. The alteration of chloride gradients in spinal cord neurons of inflamed rats, reported by several groups around the world (Aouad et al., 2014b; Morales-Aza et al., 2004; Zhang et al., 2008), could fully explain the effects of EFX seen here. In summary, these results suggest that EFX fast action in pathological pain states may be weaker than in physiological pain conditions.

Etifoxine is an interesting $GABA_A$R modulator because it has clear anxiolytic properties (Nguyen et al., 2006; Servant et al., 1998; Ugale et al., 2007) and limited adverse side effects (Micallef et al., 2001), compared to the classical benzodiazepines (e.g. amnesia, sedation, functional tolerance …). There are also growing evidences suggesting that EFX promotes nerve regeneration (Girard et al., 2008) and several molecular mechanisms have been recently proposed to explain this property (Zhou et al., 2013a, 2013b). In our laboratory, we did observe that EFX prevents the apparition of inflammatory and neuropathic pain symptoms in several pain models (Aouad et al., 2009, 2014a, 2014b). So far, we concentrated on the long-term therapeutic effects and confirmed that they were mostly mediated by the local production of the endogenous neurosteroid analgesics such as allopregnanolone (Poisbeau et al., 2014). Interestingly, the potent analgesia resulting from EFX treatment involves several synergistic mechanisms. In the spinal cord, this include a limitation of pro-inflammatory processes, a proper maintenance of chloride gradients, a protection from glycinergic disinhibition and an amplification of $GABA_A$R inhibitory function (Aouad et al., 2014b). In pathological pain states, alterations of these processes have all been demonstrated to contribute for a large part to the expression of pain symptoms (Zeilhofer et al., 2012b).

In the present study, we have characterized the fast non-genomic effects of EFX when administered in the spinal cord of rats during nociceptive processing. In this work, dedicated mostly to the electrophysiological analysis of EFX action during spinal nociceptive processing, we did not characterize potential motor or sedative side effects of the compound. Sedative effects are clearly limited in human studies when compared to similar doses of lorazepam (Micallef et al., 2001). In animal studies, they are only observed after intraperitoneal injection of concentration higher than 70 mg/kg (Poisbeau & Kamoun, unpublished data). Up to now, we have no evidence for such an effect after intrathecal injection. In addition to the *princeps* studies describing EFX potentiating effects on $GABA_A$ receptors (Hamon et al., 2003; Schlichter et al., 2000; Verleye et al., 1999, 2001), we provide here novel evidence of this mechanism, but during processing of nociceptive messages in vivo. Without having any other binding site described so far for EFX at the membrane level, our results strongly suggest that EFX reduces the spinal nociceptive processing by potentiating $GABA_A$R function at presynaptic and postsynaptic sites. In naïve rats, the inhibitory effect of EFX on the excitability of WDR neurons is not restricted to nociceptive messages mediated by Aδ and C fibers, but also affects the processing of non-nociceptive Aβ sensory messages. In monoarthritic rats, we confirmed EFX antinociception on C fiber activation threshold. In summary, the fast (present study) and long-lasting effects of EFX (Aouad et al., 2014b), mediated at least by a modulation of spinal $GABA_A$R, are of significant interest to limit spinal pain processing and the expressed pain symptoms, in physiological and pathological pain states.

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