

ORIGINAL ARTICLE

Etifoxine stimulates allopregnanolone synthesis in the spinal cord to produce analgesia in experimental mononeuropathy

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Conflicts of interest

None declared.

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Abstract

Background: Pathological pain states are often associated with neuronal hyperexcitability in the spinal cord. Reducing this excitability could theoretically be achieved by amplifying the existing spinal inhibitory control mediated by GABA_A receptors (GABAARs). In this study, we used the non-benzodiazepine anxiolytic etifoxine (EFX) to characterize its interest as pain killer and spinal mechanisms of action. EFX potentiates GABAAR function but can also increase its function by stimulating the local synthesis of 3 α -reduced neurosteroids (3 α NS), the most potent endogenous modulators of this receptor.

Methods: The efficacy of EFX analgesia and the contribution of 3 α NS were evaluated in a rat model of mononeuropathy. Spinal contribution of EFX was characterized through changes in pain symptoms after intrathecal injections, spinal content of EFX and 3 α NS, and expression of FosB-related genes, a marker of long-term plasticity.

Results: We found that a 2-week treatment with EFX (>5 mg/kg, i.p.) fully suppressed neuropathic pain symptoms. This effect was fully mediated by 3 α NS and probably by allopregnanolone, which was found at a high concentration in the spinal cord. In good agreement, the level of EFX analgesia after intrathecal injections confirmed that the spinal cord is a privileged target as well as the limited expression of FosB/ Δ FosB gene products that are highly expressed in persistent pain states.

Conclusions: This preclinical study shows that stimulating the production of endogenous analgesics such as 3 α NS represents an interesting strategy to reduce neuropathic pain symptoms. Since EFX is already prescribed as an anxiolytic in several countries, a translation to the human clinic needs to be rapidly evaluated.

1. Introduction

Control of neuronal excitability at different levels of the nociceptive system has a major impact on pain responses. In pain states, hypernociception is associated with an excess of glutamatergic transmission in many relay structures of the nociceptive system, including the spinal cord. A strategy aimed at reinforcing inhibitory controls, mediated by GABA_A receptors (GABAAR), has shown some efficacy to counteract nociceptive hyperexcitability and to reduce the associ-

ated pain symptoms (Scholz and Woolf, 2002). On the other hand, pain states have recently been associated with several mechanisms of spinal disinhibition that needs to be taken into account (Ahmadi et al., 2002; Coull et al., 2003, 2005; Harvey et al., 2004). Analgesia mediated by benzodiazepines and neurosteroids has been recently re-evaluated. Neurosteroids are of interest because some of them potentiate GABAAR function at low nanomolar concentrations (Belelli and Lambert, 2005). If analgesic properties of 3 α -reduced neurosteroids (3 α NS) are known for long time (Frye and

What's already known about this topic?

- The non-benzodiazepine anxiolytic etifoxine reduced chemotherapy-induced generalized pain symptoms.
- Etifoxine analgesia resulted from the stimulation of mitochondrial translocator protein (TSPO) which promotes neurosteroidogenesis.

What does this study add?

- Etifoxine powerful analgesia in experimental mononeuropathy is mediated by the production of allopregnanolone, at least in the spinal cord.
- Endogenous production of spinal allopregnanolone exerts an efficient acute analgesia but may also prevent long-term changes associated with chronic pain states.

Duncan, 1994), we have clearly shown that this action mostly rely on their potentiating action on GABAARs in the spinal cord. Neurosteroids can be produced by neurons and glial cells in several structures of the nociceptive system (Do Rego et al., 2009). In particular, nociceptive neurons in the superficial layers of the spinal cord (lamina I-II) express all enzymes required to synthesize 3α NS. The initiating step of neurosteroidogenesis consists of incorporating cholesterol in mitochondria before converting it into pregnenolone, the common precursor of all 3α NS. This step is ensured by translocator protein complexes (TSPO) expressed in the outer mitochondrial membrane (Rupprecht et al., 2010). Using TSPO ligands, such as benzodiazepines or benzoxazines (e.g., EFX), we have demonstrated that stimulation of neurosteroidogenesis potentiated phasic (synaptic) and tonic GABAAR functions in spinal cord neurons (Keller et al., 2004; Schlichter et al., 2006). An increased production of 3α NS has also been demonstrated in the spinal cord in a rat model of inflammatory pain (Poisbeau et al., 2005). This production reinforced GABAergic inhibitory synaptic controls and was associated with reduced pain symptoms. In line with this, the activity and expression of several neurosteroidogenic enzymes were shown to be up-regulated in various pain models (Patte-Mensah et al., 2004, 2010; Poisbeau et al., 2005; Meyer et al., 2008; Aloisi et al., 2010). The direct use of neuroactive steroids in the pain clinic has a limited interest because of its lack of specificity and of possible side effects (Smith et al., 1998; Gulinello and Smith, 2003). Promoting neurosteroidogenesis by TSPO ligands, such as EFX, may exacerbate the existing 3α NS-based homeostatic inhibitory controls, as suggested by a recent study (Aouad et al.,

2009). In this study, we first evaluated the effects of systemic administration of EFX on neuropathic pain symptoms resulting from a chronic sciatic nerve constriction in rats and demonstrated the involvement of 3α NS. This could be achieved while using finasteride and depo-provera, two inhibitors of the production of 3α NS blocking the 5α reductase and 3α -hydroxysteroid dehydrogenase, respectively (Penning et al., 1985; Stoner, 1990). In a second step, the spinal contribution of EFX action was determined using intrathecal injections, quantification of spinal EFX and 3α NS, as well as expression of FosB-related genes.

2. Materials and methods

2.1 Animals

Male Sprague-Dawley rats, weighing 150–200 g (Janvier, Le Genest St. Isle, France), were used and housed in groups of three, 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 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 PP).

2.2 Sciatic nerve constriction model of mononeuropathy

In this study, we used a rat model of peripheral mononeuropathy obtained after the surgical installation of a polyethylene cuff around the main branch of the sciatic nerve which produces a slight chronic constriction (Mosconi and Kruger, 1996; Pitcher et al., 1999). The surgical procedure was carried out under aseptic conditions and ketamine/xylazine anaesthesia (ketamine 87 mg/mL, xylazine 13 mg/mL, i.p. 10 mL/kg; Centravet, Taden, France). The right sciatic nerve was exposed and a polyethylene cuff (2-mm-long splitted section; ID = 0.86 mm, OD = 1.27 mm; PE-90, Harvard Apparatus, Les Ulis, France) was placed around it. The shaved skin layer was closed using silk suture. The sham-operated rats underwent the same surgical procedure without the cuff implantation. The animals were allowed to recover and the tests were performed 48 h after the surgery.

2.3 Evaluation of nociceptive thresholds and pain symptoms

All animals were habituated to the room, to handling and to the tests at least 1 week before starting the experiments. All tests were performed between 9:00 am and 12:00 pm, prior to any injection.

Mechanical nociceptive thresholds were measured using a calibrated forceps (Bioseb, Vitrolles, France) following a procedure previously described (Luis-Delgado et al., 2006b). Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress caused by environmental stimulations. The tips of the forceps were placed at each side of the paw and a gradually increasing force was applied. The pressure producing withdrawal of the paw or the vocalization of the animal corresponded to the nociceptive threshold value. This manipulation was performed three times for each hindpaw and the values were averaged.

Thermal cold allodynia was determined by scoring the aversive behaviours of rats using the acetone test (Choi et al., 1994; Flatters and Bennett, 2004). Rats were placed in a Plexiglas cage on a wire mesh and allowed to accommodate for at least 15 min. A drop of acetone was then placed on the ventral side of the hind paw. The evaporation of the acetone drop produces a decrease in temperature, a stimulus that is non-noxious for the rat. The animal behavioural response was monitored during 20 s after the acetone application and scored as follows: 0, no response of the animal; 1, quick withdrawal, flick or stamp of the paw; 2, prolonged withdrawal or repeated flicking of the paw; 3, repeated flicking of the paw with licking of the paw. This procedure was performed three times for each paw and the values were added. In control animals, acetone score was always found to be below 1, whereas in allodynic animals, it can reach the maximal value of 9.

2.4 Drugs

For the behavioural pharmacology experiments, each animal group was composed of six adult male rats. Three different experiments were performed after initial characterization of the sciatic nerve injury model and experimenters were blinded to the different treatment conditions.

2.4.1 Dose-dependent therapeutic effect of etifoxine

Etifoxine (EFX; 2-ethylamino-6-chloro-4-methyl-4-phenyl-4*H*-3,1-benzoxazine hydrochloride; batch 523; Biocodex, Gentilly, France) was prepared in saline (NaCl 0.9% in distilled water) containing 1% Tween 80 (v/v; Sigma, St Louis, MO, USA). Different concentrations (1, 5, 25 and 50 mg/kg) were used and injected i.p. in a final volume of 10 mL/kg. Animals from the control group received an equivalent volume of vehicle (0.9% NaCl in distilled water with 1% Tween 80 (v/v)). In this study, we analysed the effects of EFX treatments on neuropathic pain symptoms (i.e., 2 and 4 weeks after cuff implantation). Daily measures of cold allodynia and mechanical hyperalgesia were always performed before any injection of EFX (e.g., >17 h after each injection to prevent any GABAAR-related mechanisms).

2.4.2 Contribution of neurosteroids to etifoxine therapeutic effect

To characterize EFX action through the possible synthesis of neuroactive neurosteroids, we used two inhibitors of neurosteroidogenesis: (1) Provera (PRO: 6-medroxyprogesterone acetate; Steraloids, London, UK) is a pharmacological inhibitor of the 3 α -hydroxysteroid oxidoreductase (3 α HSOR) responsible for the production of the 3 α NS, which are potent allosteric modulators of GABA_A receptor channels; and (2) Finasteride [FIN: 5 α ,17 β -*N*-(1,1-dimethylethyl)-3-oxo-4-azaandrost-1-ene-17-carboxamide; Steraloids] is a pharmacological inhibitor of the 5 α reductase (one step upstream of 3 α HSOR) responsible for the synthesis of 5 α -reduced neurosteroids. PRO was suspended in olive oil and subcutaneously administered (1 mg/kg every 2 days; injected volume: 1 mL/kg). Fin was injected subcutaneously at 30 mg/kg in olive oil (three times a week; injected volume of 1 mL/kg). Control animals for PRO and FIN experiments received an equivalent volume of olive oil. PRO and FIN treatments started 1 week before the cuff surgery and lasted for 3 weeks.

2.4.3 Spinal cord as a target for etifoxine action

In a subset of experiments, EFX [diluted in 0.9% NaCl containing 30% (v/v) ethanol] was administered directly in the spinal cord (intrathecal injection) at a concentration of 0.6 μ g/20 μ L. In this case, EFX vehicle [0.9% NaCl containing 30% (v/v) ethanol] was used for control experiments.

2.5 FosB/ Δ FosB immunohistochemistry

Immunohistochemistry was performed on a separate set of animals ($n = 6$ /group) treated with etifoxine, used at two different concentrations: analgesic (50 mg/kg) and not analgesic (control: 1 mg/kg). On day 21 (e.g., after the 2 weeks EFX treatment), rats were perfused intracardially with 150 mL of phosphate buffer (PB: 0.1 mol/L, pH 7.4) followed by 500 mL of 4% paraformaldehyde/0.6% picric acid in phosphate buffer (0.1 mol/L, pH 7.4). The lumbar spinal cord (L2–L5) were removed and post-fixed overnight with the same fixative and washed the next day in phosphate buffer saline (PBS). The lumbar spinal cord was notched on the ventral right side to allow both sides to be distinguished. Transverse sections of 50 μ m were cut on a vibratome (VT1000S, Leica, Wetzlar, Germany) and incubated in a blocking solution of 5% donkey serum in PBS Triton X-100 (0.5%) for 1 h. The sections were then incubated overnight with a 'pan-FosB' antibody raised against an internal region of FosB recognizing both FosB and Δ FosB proteins (rabbit polyclonal 1:2000 recognizing the FosB C-terminal region) in 1% donkey serum in PBS-Triton 0.5% at room temperature. After a wash with PBS, sections were incubated 90 min with the secondary biotinylated anti-rabbit IgG (1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). They were then treated with a peroxidase-conjugated avidin-biotin

complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. ABC reaction was Tris buffer (pH revealed by incubation in 250 mg/mL diaminobenzidine, 0.03% H₂O₂ in 0.05 mL Tris buffer (pH 7.6) and 5 mg/mL nickel ammonium sulphate at room temperature. The sections were rinsed once with Tris buffer (pH 7.6) and twice with PBS before being mounted on gelatinized slides. Analysis of the FosB/ Δ FosB expression was performed blinded. All immunopositive nuclei were counted manually on randomly chosen lumbar (L1–L6) spinal cord sections with intact right and left dorsal horns (3 per animal; $n = 6$ rats/group) using 10 \times objective on a Leica Leitz DMRD microscope. While comparing ipsilateral and contralateral side counts, we found no differences between superficial (laminae I–II) and deep layer (laminae III–V) and data were pooled to express the difference (ipsi-contralateral) in immunopositive nuclei per spinal cord slice. Two independent experimenters were involved in this analysis and had similar results.

2.6 Etifoxine and neurosteroid dosage

Dosage of neurosteroids was carried out using spinal cords of rats exhibiting pain symptoms after cuff implantation and treated with growing doses of etifoxine (Fig. 1C and D). Animals were sacrificed by decapitation 3 days after the last etifoxine injection. The lumbar spinal cord was quickly removed and stored at -80°C . For EFX and steroid extraction, the spinal cords were first homogenized in 1 and 2 mL of water, respectively. Two volumes of methanol were added and the samples were centrifuged at 14,000 rpm for 20 min at 4°C . The supernatant was removed and evaporated to dryness in a speedvac concentrator (Thermo Fisher Scientific, Illkirch Grafenstaden, France). Dry extracts were kept at -20°C before use for high pressure liquid chromatography (HPLC) analysis. Reversed-phase HPLC was performed on Macherey-Nagel Nucleosil columns (RP300 5C18, Hoerdt, France) mounted on an Akta Purifier (GE Healthcare, Paris, France). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid in water (buffer A) and 0.09% (v/v) trifluoroacetic acid and acetonitrile 70% in water (v/v; buffer B). HPLC elutions were performed at a flow rate of 700 $\mu\text{L}/\text{min}$ using the following gradient: 0–60% of buffer B in 10 min and then 60–90% in 30 min. Absorbance was monitored at 202 nm in order to quantify allopregnanolone (THP: 5 α -pregnan-3 α -ol-20-one) and allo-tetra-hydro-deoxy-corticosterone (THDOC: 5 α -pregnan-3 α ,21-diol-20-one). All compounds were purchased from Steraloids except EFX, which was provided by Biocodex.

2.7 Statistics

All data are expressed as mean \pm standard error of the mean. One- or two-way repeated-measures analysis of variance (ANOVA), followed by Tukey's post hoc tests (Statistica, StatSoft, Tulsa, OK, USA), were used to analyse the effects on mechanical sensitivity. To analyse the ordinal data from the

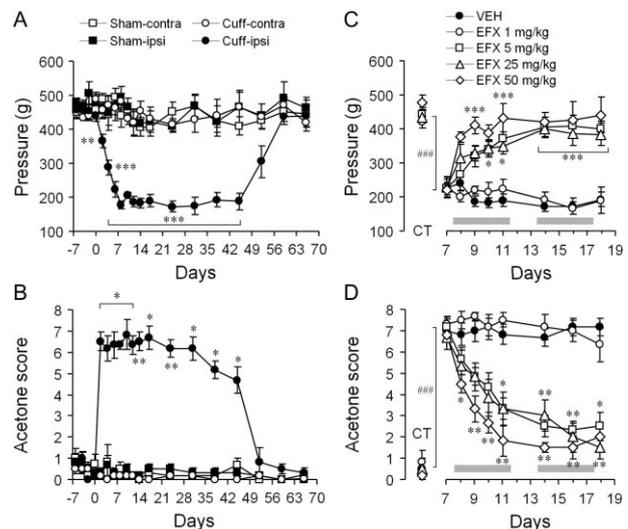


Figure 1 (A, B) Time course of mechanical nociceptive threshold (A) and behavioural scores following acetone application (B) for sham-operated and cuffed rats ($n = 6/\text{group}$). Day 0 indicates day of the surgery for the sciatic nerve cuffing. Two-way analysis of variance with repeated measures on mechanical thresholds ($F_{18,360} = 4.56; p < 0.0001$) and Friedman's test on acetone scores ($\chi^2_{18} = 41.58; p < 0.001$) of cuffed rats showed significant effects of the interaction between time, side and surgery. (C, D) Evolution of the hyperalgesic pressure thresholds (C) and cold allodynic scores (D) of the cuffed paw before (day 7) and after i.p. injection with etifoxine (EFX 1, 5, 25, 50 mg/kg, $n = 6$ rats/group) or its vehicle (VEH). Treatments consisted of 10 injections (2 series of 5 injections, 1 injection/day; treatment period shown in grey) and started at the end of day 7. Baseline mean pressure threshold and acetone score are indicated on the graph (CT: before surgery) for each group. Compared with the value obtained at day 7, etifoxine treatments with 5, 25 and 50 mg/kg i.p. significantly reduced mechanical hyperalgesia ($F_{32,200} = 3.66; p < 0.001$) and cold allodynia ($\chi^2_8 = 111.99; p < 0.001$). At each time point and compared with contralateral paw (panels A and B) or vehicle-treated animals (panels C and D), asterisks indicate statistical significance, while comparing mechanical thresholds (Tukey's test) and acetone scores (Wilcoxon). Significance code: * $p < 0.05$; ** $p < 0.01$; *** or **** $p < 0.001$ (D7 vs. CT).

acetone scoring test, non-parametric test (Friedman test) followed by Wilcoxon's or Dunn's post hoc tests were used (Statistica, StatSoft). Immunochemistry data were analysed using two-tailed Student's t -test for paired data. Differences were considered to be statistically significant for $p < 0.05$.

3. Results

3.1 EFX effects on neuropathic pain symptoms

As illustrated in Fig. 1 (panels A and B), cuff-implanted rats rapidly exhibited pain symptoms at the ipsilateral (cuffed) hindpaw. Mechanical hyperalgesia was characterized by an important decrease in mean pressure threshold from 440.4 ± 17.7 g before the surgery (day 0) to 177.0 ± 10.2 g, 1 week (day 7) after

the surgery ($n = 6$ rats/group; Fig. 1A). Neuropathic pain lasted for 7–8 weeks. Cold allodynia appeared rapidly after implantation of the cuff. After acetone stimulation of the cuffed hindpaw, the behavioural score reached values of 6.3 ± 0.2 ($n = 6$), whereas it was always below 1 in sham-operated animals and after testing of the contralateral hindpaws. As shown in Fig. 1, chronic constriction of the sciatic nerve by the cuff was associated with pain symptoms lasting 7–8 weeks for mechanical hyperalgesia (panel A) and cold allodynia (panel B). This is fully consistent with the original study describing the model of sciatic nerve cuffing (Mosconi and Kruger, 1996).

To characterize etifoxine (EFX) effects on mechanical hyperalgesia and cold allodynia, cuff-implanted rats were first treated with different doses of EFX (1, 5, 25 and 50 mg/kg, i.p.). EFX or its vehicle (VEH) was always administered to rats at the end of the nociceptive tests and treatment started 1 week (at day 7) after the surgery, i.e., when the intensity of pain symptoms was maximal and stable. In this study, EFX treatment was maintained for 2 weeks (see the Materials and methods section for details). At a concentration of 1 mg/kg, no analgesic effect of EFX was seen on pain symptoms (Fig. 1C and D). Indeed, minimal mechanical pressure thresholds and elevated behavioural scores were not different from those measured in vehicle-treated animals (Fig. 1C and D). In our hands, EFX treatment, at doses of 5, 25 or 50 mg/kg, proved its efficacy by progressively reducing mechanical hyperalgesia (Fig. 1C) and cold allodynia (Fig. 1D) after one to two injections. The analgesic effect of EFX was maintained all along the treatment. After a 1-week treatment, pain symptoms were no longer detected as indicated by the lack of statistical difference in the pain thresholds/scores of treated-animals compared with their values before sciatic nerve injury (Fig. 1C and D, control CT).

If a maximal analgesic effect is observed at a low dose of 5 mg/kg, treatment of rats with 50 mg/kg was associated with a faster recovery. The significant anti-hyperalgesic and anti-allodynic efficacy was indeed reached at day 9 and at day 11 for the respective doses of 50 and 5 mg/kg, for mechanical pressure thresholds and acetone scores (data not shown). For all efficient regimens (5–50 mg/kg), EFX analgesia persisted until the full recovery of vehicle-treated animals (and of rat treated with 1 mg/kg).

In a separate experiment, we also evaluated the efficacy of EFX to treat pain symptoms at a later stage of the neuropathic period (Fig. 2). Administration of EFX at a dose of 25 mg/kg, 4 weeks after the cuff implantation, displayed a similar efficacy. As indicated

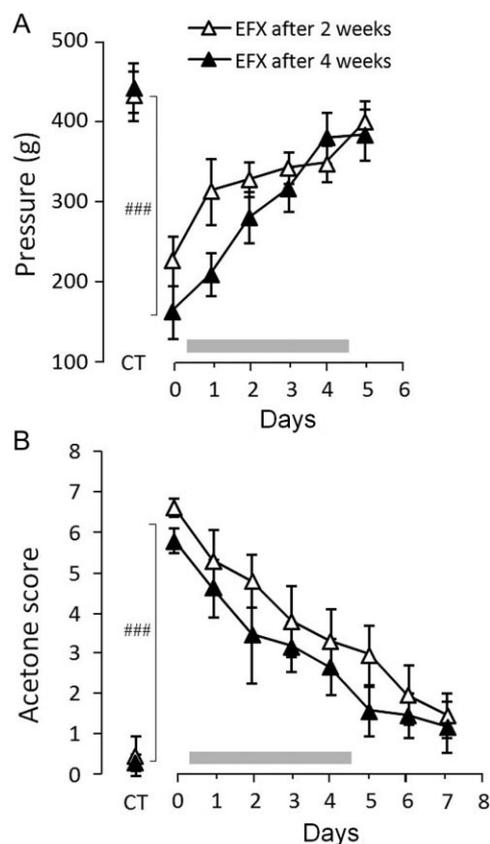


Figure 2 (A, B) Changes in the hyperalgesic pressure thresholds (A) and cold allodynic scores (B) of the cuffed paw before (day 7) and after i.p. injection with etifoxine (EFX 25 mg/kg, $n = 6$ rats/group). The graph illustrates the anti-hyperalgesic and anti-allodynic effect of EFX when administered 2 weeks (open symbols) and 4 weeks (black symbols) after initiation of neuropathy (cuff surgery). Treatment consisted of five injections (1 injection/day; treatment period shown in grey) and started at day 0. Nociceptive tests were carried out prior to any injections (> 17 h after each injection). Baseline mean pressure threshold and acetone score are indicated on the graph (CT: before surgery) for each group. No differences could be found while comparing the group values at each time point. Statistical significance was reached ($***p < 0.001$) while comparing values at day 0 and control (CT) for each group.

in the figure, the reduction in mechanical hyperalgesia (panel A) and cold allodynia (panel B) had a similar time course during the treatment week.

3.2 Contribution of endogenous neurosteroid to the therapeutic effect of EFX

The known mechanisms by which EFX exert its action relies on the positive modulation of GABAAR-mediated inhibitory function. In addition to the described direct allosteric modulation of the receptor, there is growing evidence indicating that EFX exerts

its action by stimulating the local production of 3α NS (e.g., allopregnanolone, THDOC), known as the most potent endogenous modulator of GABAAR function. In this study, we failed to reveal the presence of EFX by HPLC in the nervous system (spinal cord, encephalon; not shown) of our treated rats, 24 h after the end of the treatment. This fitted well with the known half-life of EFX and strongly suggested that EFX action on GABAARs could be indirect and mediated by the stimulation of endogenous neurosteroid production. We therefore determined the contribution of this mechanism in our model of mononeuropathy. Rats were pre-treated (1 week before surgery) with subcutaneous injections of FIN (30 mg/kg; every 2 days) or PRO (1 mg/kg; every 2 days), two inhibitors of neurosteroidogenesis inhibiting 5α -reductase and 3α -hydroxysteroid oxidoreductase activities, respectively. The treatment with PRO and FIN was maintained after cuff implantation and during the treatment with EFX (25 mg/kg, i.p., starting at day 7). As shown in Fig. 3, the therapeutic effect of EFX could only be observed in the FIN/PRO-vehicle (olive oil s.c.; Fig. 3, VEH) group of rat, treated with the vehicle and having a preserved capability of synthesizing 3α neurosteroids. In this control group, after 10 injections of EFX (25 mg/kg, i.p.), mean pressure threshold values (Fig. 3A: 454.8 ± 24.6 ; $n = 6$) and behavioural scores to acetone (Fig. 3B: 1.0 ± 0.5 ; $n = 6$) were not statistically different from that of pain-free animals (see above). In sharp contrast, EFX failed to diminish pain symptoms in rats pretreated with PRO or FIN. Animals from these two groups exhibited low pressure thresholds (FIN-treated: 293.8 ± 31.5 g, $n = 6$; PRO-treated: 281.4 ± 33.4 g, $n = 6$) and elevated acetone score (FIN-treated: 6.7 ± 0.9 , $n = 6$; PROV-treated: 6.3 ± 0.6 , $n = 6$), indicating that EFX likely exerted its analgesic action through the stimulation of 3α neurosteroid synthesis.

Although 3α neurosteroids are difficult to quantify because of their expected very low concentrations, we performed HPLC dosage for two of them, allopregnanolone (THP) and THDOC in the spinal cord (Fig. 4), 3 days after the end of a 5-day EFX treatment, aimed at stimulating their concentration. As mentioned earlier, EFX remained undetected in all tissues at this time point, indicating that its analgesic effect could likely be mediated by a long-lasting tonic stimulation of neurosteroidogenesis.

At 1 mg/kg (or even after VEH injection), we did not find any evidence for the presence of THP or THDOC (see representative example in Fig. 4). At an efficient therapeutic dose (EFX 5–50 mg/kg), low amounts of allopregnanolone in the spinal cord of animals were

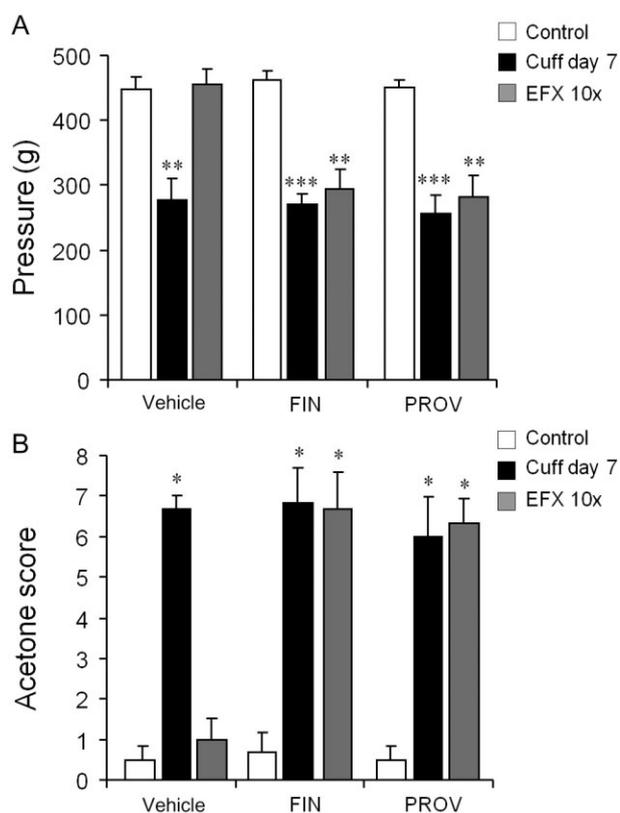


Figure 3 Histograms illustrating the mean mechanical threshold (A) and the acetone behavioural score (B) of cuffed rats ($n = 6$ rats/group) after the simulation of the ipsilateral hindpaw, before the surgery (control), 7 days after surgery (cuff day 7) and after 10 daily injections of EFX (EFX 10x). One group of rats was pretreated with Finasteride (FIN) (30 mg/kg, s.c.; black bars) and Provera (PRO) (5 mg/kg, s.c.; grey bars). Control values were obtained by injection of the vehicle (VEH, olive oil, s.c.; white bars). A significant interaction between treatment (VEH, FIN, PROV) and time (Control, cuff day 7, EFX 10x) was found while comparing mechanical thresholds (analysis of variance: $F_{4,30} = 4.347$, $p < 0.01$) and allodynic scores (Friedman: $\chi^2_2 = 23.41$; $p < 0.001$). Asterisks indicate statistical significance compared with the control values for each group, using post hoc tests for pressure values (Tukey) and behavioural scores (Wilcoxon). Significance code: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

observed. Treatment with 5 mg/kg of EFX was associated with spinal allopregnanolone levels of 27.8 ± 6.9 pg/mg of protein ($n = 6$ rats) and this corresponded to a local concentration of about $1 \mu\text{mol/L}$. This concentration was even higher after EFX treatment with 25 mg/kg (255.6 ± 87.0 pg/mg of protein, $n = 4$) and 50 mg/kg (453.9 ± 83.4 pg/mg of protein, $n = 4$). Interestingly, we failed to detect THDOC in our samples (detection limit 30 pg/sample). Note that allopregnanolone was detectable in our hands only above 6 pg/sample.

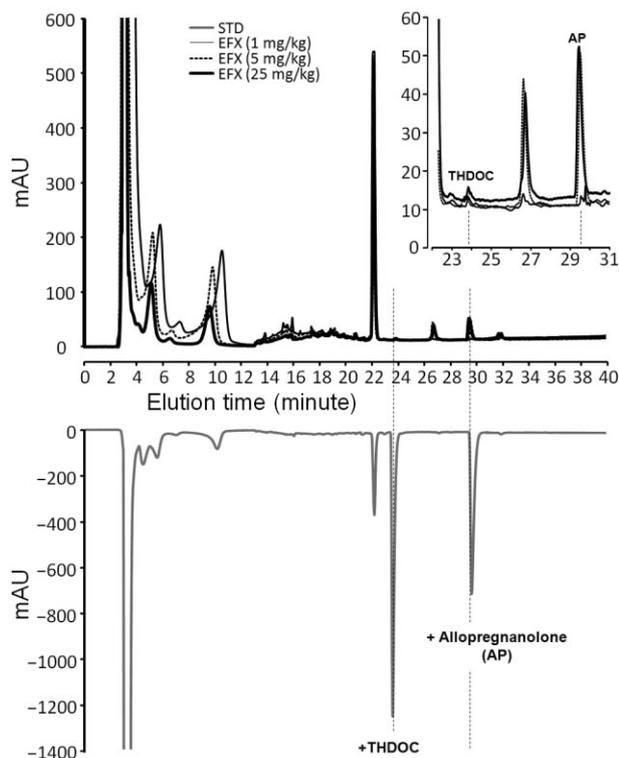


Figure 4 Chromatograms in the upper graph illustrate the detection of THDOC (5 α -pregnan-3 α ,21-diol-20-one) and allopregnanolone (AP) in spinal cord samples taken from cuffed rats treated with 1 (thin line), 5 (dashed line) and 25 mg/kg i.p. (thick line). A comparison is made with the chromatogram (bottom graph) obtained with spinal cord samples collected from untreated cuffed rats but containing known concentration of THDOC (53 nmol) and AP (114 nmol). This allowed identifying peaks corresponding to THDOC and AP in order to quantify contents. In the upper graph, the inset shows a magnification of the peaks corresponding to the elution time of THDOC and allopregnanolone. Note that only allopregnanolone content was increased.

3.3 Spinal contribution to EFX analgesia

To further investigate the action of EFX and the contribution of spinal circuits to its effect, EFX was injected intrathecally (0.6 μ g/20 μ L) in neuropathic animals and mechanical pressure thresholds were measured. As illustrated in Fig. 5A, the first injection of EFX produced a significant but transient anti-hyperalgesic effect on mechanical pressure thresholds. This effect was maximal 1 h after the intrathecal injection but returned to basal level after 4 h. As indicated, no significant changes were seen after injection of EFX or of the vehicle on the contralateral hindpaw mean values. Interestingly, 24 h after the first injection, mechanical thresholds were significantly increased (from 206.8 \pm 7.6 to 258.8 \pm 8.0 g at 24 h, n = 6). This effect was maintained and stable thereafter and corre-

sponded to about 35% reduction in mechanical hyperalgesia. This indicated that even after one injection of EFX, a prolonged antinociceptive effect can be seen.

Neuropathic pain is a chronic state that can lead to major modifications of the nervous system circuits in the spinal cord. In line with this basic statement, FosB/ Δ FosB was previously proposed as an interesting marker of plasticity associated with neuronal hyperexcitability. This includes studies performed on the spinal cord of rats exhibiting a painful inflammatory pain sensitization (Luis-Delgado et al., 2006a). In order to establish a possible neuroprotective role of EFX against this molecular plasticity in the spinal cord,

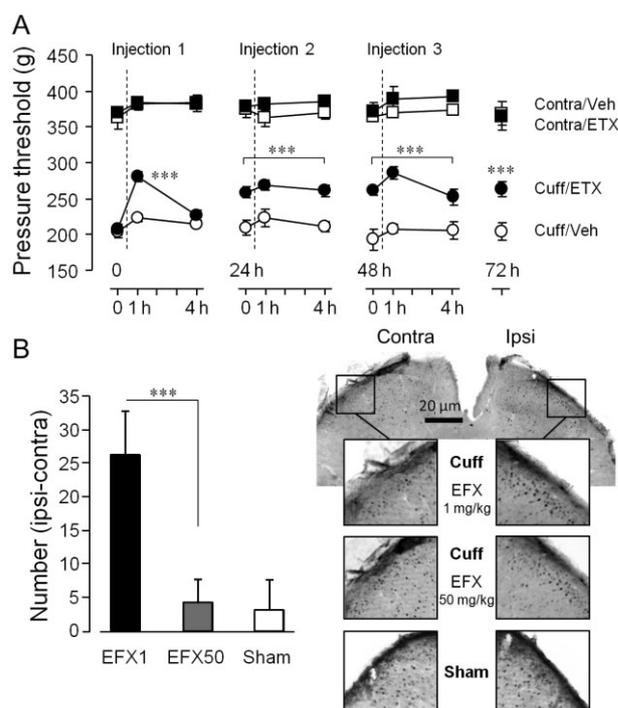


Figure 5 (A) Time course of mechanical nociceptive threshold for rats (n = 6/group) receiving intrathecal injection of EFX (black symbols) or of its vehicle (open symbols). Values obtained after stimulation of the contralateral and ipsilateral hindpaw are indicated by squares and circles, respectively. Two-way repeated measures analysis of variance revealed a significant statistical effect of the time ($F_{9,180}$ = 3.18; p < 0.05) and of the interaction between side and treatment ($F_{1,20}$ = 25.56; p < 0.05). Asterisks indicates significance ($***p$ < 0.01) after Tukey post hoc tests while comparing corresponding time points for the cuff group, treated with EFX or vehicle. Note that values obtained for the contralateral paws (EFX or VEH group) were statistically different compared with those obtained from the ipsilateral paws (not indicated but p < 0.001). (B) Histogram illustrating differences between ipsilateral and contralateral dorsal horn while counting FosB/ Δ FosB immunopositive nuclei for 1 and 50 mg/kg regimen of EFX after 10 injections. Counts and images for sham values are also shown. Asterisks indicate significance at p < 0.001 using two-tailed Student's t -test with paired data.

we performed immunohistochemistry for FosB/ Δ FosB in neuropathic rats treated by an inefficient (1 mg/kg) and efficient analgesic regimen of EFX (50 mg/kg). After a 12-day treatment, we found FosB/ Δ FosB immunoreactivity to be more expressed in the ipsilateral (grey matter) dorsal horn of the lumbar spinal cord for animals treated with 1 mg/kg EFX (Fig. 5: $n = 6$ rats/group). The number of positive FosB/ Δ FosB positive nucleus was on average 1.59 ± 0.09 times more numerous in the ipsilateral dorsal horn ($n = 411$ nucleus; $n = 6$ rats) compared with the respective contralateral side ($n = 254$ nucleus; $n = 6$ rats). A similar finding was noted in vehicle-treated animals (not shown). For the effective treatment with 50 mg/kg of EFX, the number of positive FosB/ Δ FosB nucleus was lower and found to be equally represented in both sides of the dorsal horn (ratio: 1.10 ± 0.08 ; contralateral, $n = 342$ nucleus; ipsilateral, $n = 368$ nucleus; $n = 6$ rats/group). We failed to reveal any difference between deep and superficial layers/ which exhibited a similar asymmetry of FosB/ Δ FosB immunopositive nuclei in the case of the non-efficient regimen of 1 mg/kg EFX. It should be noticed, however, that FosB/ Δ FosB positive nuclei were far more numerous in the most superficial layers as shown in the figure.

4. Discussion

The first aim of this study was to characterize the effect of EFX on pain symptoms generated by chronic constriction of the sciatic nerve. In the second part, we showed that EFX analgesic action is ensured by the local production of analgesic neurosteroids, such as allopregnanolone, in the spinal cord. This production likely explained the reduced spinal hyperexcitability and long-term plasticity observed in this pain condition.

Anxiolytic properties of EFX have been described in rodents (Boissier et al., 1972) and in human (Servant et al., 1998; Nguyen et al., 2006). Interestingly, EFX treatment of human anxiety was not associated with sedative, myorelaxant and amnesic side effects (Servant et al., 1998; Micallef et al., 2001). Using rat models, EFX significantly reduced stress-induced hyperthermia and freezing behaviours following fear conditioning, 60 min after i.p. injection of EFX only at a concentration >50 mg/kg (Verleye and Gillardin, 2004). A similar concentration produced significant anxiolysis in the Vogel's conflict test (Verleye et al., 2005). Anxiolytic effects of EFX was also achieved shortly after injection of lower doses (<10 mg/kg, i.p.) in mice (Verleye et al., 2003). In summary, these

studies and some others clearly indicated that EFX produces an efficient anxiolysis and seizure protection.

Recently, we demonstrated for the first time that EFX displayed important analgesic properties on generalized neuropathic pain symptoms induced by the anti-tumoural agent vincristine sulfate in the rat model (Aouad et al., 2009). However, our experimental approach strongly differed from the studies cited above because animal testing (for nociception here) was carried out systematically before any injection (i.e., at least 17 h after each daily EFX injection). In line with this study, our present result not only demonstrates that a curative treatment with EFX abolish pain symptoms evoked by a chronic constriction of the sciatic nerve, but also reinforce the conclusion that EFX had long-lasting neuroprotective benefits in neuropathic states. We also showed here that a minimal dose of 5 mg/kg i.p. was required for rat. A similar conclusion appeared recently in a study characterizing the effects of EFX on functional recovery of rat locomotion and motor coordination after sciatic nerve cryo-lesion (Girard et al., 2008). This action, associated with an acceleration of nerve regeneration, was seen at a unique dose of 50 mg/kg (i.p.) and it is not excluded that a lower dose might have been efficient.

The use of selective inhibitors of neurosteroidogenesis (FIN and PRO) led us to conclude that 3α NS are the sole endogenous compounds responsible for the reduction of neuropathic pain symptoms as FIN and PRO totally abolished EFX-induced analgesia. Allopregnanolone-like neurosteroids seemed also to mediate anxiolytic effects of EFX, as indicated by two independent studies using Vogel's conflict test (Verleye et al., 2005) and elevated plus maze test in rats (Ugale et al., 2007). These two lines of evidence supported the idea that therapeutic action of EFX stimulates the local synthesis of 3α NS, which could exert their action by potentiating GABAARs (Schlichter et al., 2000; Hamon et al., 2003), blocking T-type calcium channels (Pathirathna et al., 2005) or by other (still unidentified) means. To support this idea, we failed to detect EFX in the spinal cord of treated rats, 3 days after the end of the treatment, but we did find low amounts of allopregnanolone (and of some precursors) in the spinal cord of EFX-treated animals. This was not a surprising result because EFX was previously shown to be cleared rapidly after i.p. injection (Verleye et al., 2005, 2010). Immediately after injection of 50 mg/kg i.p. in rats, EFX concentrations reached a concentration of 6 μ g/mL and 11 μ g/g in the plasma and brain, respectively. The half-life of EFX for both tissues could be estimated to be about 60–90 min (data confirmed provided by Biocodex laboratories). This strongly sug-

gested that EFX exerts a triggering action on TSPO and, if administered at an efficient concentration (>5 mg/kg, i.p.), promote a long-lasting production of analgesic neurosteroids. As indicated by our experiments using a range of EFX concentrations, the final concentration of neurosteroids has presumably to be above a given concentration window to produce an all-or-none therapeutic action. Dosage of various steroids in the spinal cord strongly suggested allopregnanolone to exert an important contribution to the reduction of neuropathic pain symptoms. A similar conclusion was reached in the case of anxiolysis produced shortly (30 min) after i.p. injections of EFX at 50 mg/kg (Verleye et al., 2005). In this experiment and compared with the appropriate controls, allopregnanolone levels were more than doubled in the brain of EFX-treated rats, regardless of the presence of adrenals. Unfortunately, there are no available data to characterize clearance of neurosteroids in nerve tissues after stimulation by ETX. Alternatively, the initial therapeutic action and the associated neuroprotection might have been sufficient to accelerate recovery/adaptation in this chronic constriction injury model, as proposed in another model of nerve lesion (Girard et al., 2008). In agreement with this, other TSPO ligands were recently shown to exert a significant neuroprotection in experimental models of cytotoxic neuropathy (Giatti et al., 2009). Further studies will be required to identify the molecular mechanisms involved, but mitochondrial TSPO complexes are definitely the key players in these processes (Mills et al., 2005; Rupprecht et al., 2010). TSPO complexes are, e.g., overexpressed in the ipsilateral spinal cord of rodents with monoarthritic (Hernstadt et al., 2009) or after sciatic nerve transection (Karchewski et al., 2004) as well as in platelets of fibromyalgic patients (Bazzichi et al., 2006). This overexpression, if it exists in this model, may explain the analgesic efficacy of ETX in our study.

Neurons located in the dorsal horn of the spinal cord are the first central relay for peripheral nociceptive messages. Moreover, they express the enzymes leading to the production of allopregnanolone-like neurosteroids (Schlichter et al., 2006). We have previously shown that modulation of this endogenous synthesis finely tunes GABAergic inhibition during development and at more adult stages (Keller et al., 2004; Poisbeau et al., 2005). Moreover, allopregnanolone-like compounds, when injected in the spinal cord, are potent analgesics (Frye and Duncan, 1994; Charlet et al., 2008; Meyer et al., 2008). Therefore, EFX might preferentially exert its action in the spinal cord. We attempted to evaluate this by injecting small volumes of

EFX to rat exhibiting mechanical hyperalgesia after 1 week of implantation of a cuff (Fig. 5). After the first injection, a significant but transient antihyperalgesia could be observed likely indicating a direct allosteric action of EFX on fast GABAergic inhibition. After the first injection, transient antihyperalgesia was no longer observed, but mechanical nociceptive thresholds were progressively increased after three injections, thus contributing to approximately 35% of analgesia compared with vehicle-injected animals. This effect is quite similar to what was observed after intrathecal injection of 1–10 $\mu\text{mol/L}$ of allopregnanolone in allodynic rats (Charlet et al., 2008). In parallel to the quantification of pain symptoms, we used FosB/ Δ FosB immunolabelling to reveal the long-lasting hyperactivation of spinal neurons in pain states. Contrary to other Fos protein members, FosB-like proteins accumulate and seem to be a good marker of neuronal excitability, especially in persistent adaptation of brain responses to chronic perturbations (McClung et al., 2004). This marker was, e.g., overexpressed in the ipsilateral spinal cord of rats following intraplantar carrageenan inflammation (Luis-Delgado et al., 2006a). In the present study, FosB/ Δ FosB immunoreactivity was 1.6 times more important in the dorsal horn of the spinal cord of hyperalgesic rats treated with 'non-therapeutic' dose of EFX at 1 mg/kg. Overexpression was no longer observed while using the spinal cord of rats treated by EFX at 50 mg/kg, a regimen that fully restored normal mechanical nociception thresholds and prevented the appearance of aversive behaviours to acetone stimulation. So far, we have no evidence indicating that microglial or astroglial cells express these markers in pain states, but, since we did not performed double labelling of these cells, we cannot exclude their contribution to FosB/ Δ FosB immunoreactivity and a possible analgesic action of EFX mediated by these cells. Future studies will also be required to characterize the supraspinal contribution of EFX to the global analgesia. Moreover, novel molecular targets contributing to its neuroprotective action need to be identified in order to confirm that EFX could be a promising pain relief alternative.

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Author contributions

M.A.: Pain symptom measurements, pharmacological treatment HPLC dosage, immunohistochemistry, data analysis, paper drafting and commenting.

N.P.-D.: Pain symptom measurements, pharmacological treatment.

Y.G.: Supervision and analysis of HPLC dosage, paper commenting.

P.P.: Experimental design and supervision, data analysis, paper writing.

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