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The anxiolytic etifoxine activates the peripheral benzodiazepine receptor and increases the neurosteroid levels in rat brain

Marc Verleye [a,*], Yvette Akwa [b], Philippe Liere [b], Nathalie Ladurelle [b], Antoine Pianos [b], Bernard Eychenne [b], Michael Schumacher [b], Jean-Marie Gillardin [a]

[a] *Département de Pharmacologie, Biocodex, Zac de Mercières, Chemin d'Armancourt, 60200 Compiègne, France*
[b] *INSERM U488, Le Kremlin-Bicêtre, France*

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Abstract

The peripheral benzodiazepine receptors (PBR) might be involved in certain pathophysiological events, such as anxiety, by stimulating the production of neuroactive steroids in the brain. A recent electrophysiological study has revealed an interaction between PK11195, a PBR ligand and the anxiolytic compound etifoxine at micromolar concentrations. The present work was aimed at further characterizing the etifoxine–PBR interaction. In membrane preparations from intact male rat forebrain, etifoxine uncompetitively inhibited the binding of [^{3}H]PK11195 with an $IC_{50} = 18.3 \pm 1.2$ μM, a value consistent with etifoxine plasma and brain concentrations measured after an anxiolytic-like dose (50 mg/kg). In vivo, that etifoxine dose was associated with increased concentrations of pregnenolone, progesterone, 5α-dihydroprogesterone and allopregnanolone in plasma and brain of sham-operated animals. In adrenalectomized and castrated rats, etifoxine enhanced the brain levels of these steroids, suggesting a stimulation of their local synthesis and/or a decrease of their disappearance rate, independently of peripheral sources. Finasteride, an inhibitor of 5α-reductase that converts progesterone into its 5α-reduced metabolites like allopregnanolone, attenuated the anti-conflict effect of etifoxine even though brain allopregnanolone contents were drastically reduced. These results indicate that following activation of the PBR in the brain, an increased cerebral production of allopregnanolone, a potent positive modulator of the $GABA_A$ receptor function, may partially contribute to the anxiolytic-like effects of etifoxine.

Keywords: Etifoxine; Peripheral benzodiazepine receptor; Neurosteroids; Allopregnanolone; Plasma and brain etifoxine levels; Adrenalectomized-castrated rats; Finasteride; Anxiety

1. Introduction

A variety of steroids named "neurosteroids" may be synthesized within the brain from cholesterol, independently of peripheral endocrine sources (Baulieu, 1991). Among them, some are neuroactive regulating diverse brain functions in rodents. Allopregnanolone, the 3α,5α-reduced metabolite of progesterone, displays potent anxiolytic (Bitran et al., 1991; Brot et al., 1997) and anticonvulsant (Belelli et al., 1989) properties, and also impairs memory performance (Ladurelle et al., 2000). The actions of neurosteroids at the membrane level have been extensively studied. One of the best-documented example is the activation of $GABA_A$ receptors by allopregnanolone (Harrison and Simmonds, 1984; Lambert et al., 1999) and the anti-conflict effect of allopregnanolone has been shown to be mediated by these receptors, independently of the classical benzodiazepine site (i.e. flumazenil site) (Brot et al., 1997). The pharmacological activities of neurosteroids also implicate other neurotransmitter receptors including glutamate, nicotinic, acetylcholine and 5-HT3 receptors (Lambert et al., 1999; Rupprecht et al., 2001). In addition, the peripheral (mitochondrial) type benzodiazepine receptor (PBR) is known to play an important role in regulating the central and peripheral synthesis of neuroactive steroids (Papadopoulos, 1993; Papadopoulos et al., 2001). Recently, it has been shown that newly synthesized selective PBR agonists (2-phenyl-imidazo[1,2-a]pyridine derivatives and FGIN 1-27) enhanced the production of allopregnanolone associated with marked anxiolytic-like effects in the rat (Serra et al., 1999; Bitran et al., 2000).

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* Corresponding author. Tel.: +33 3 44 86 82 28; fax: +33 3 44 86 82 34.
E-mail address: m.verleye@biocodex.fr (M. Verleye).

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Etifoxine, a molecule chemically unrelated to benzodiazepines (BZDs), has anxiolytic-like properties in rodents (Boissier et al., 1972; Verleye and Gillardin, 2004) and is effective for treating adjustment disorder with anxiety in humans (Servant et al., 1998). The mechanism of action of etifoxine is not completely understood, but some data suggest that it may enhance GABAergic transmission by a direct allosteric effect on $GABA_A$ receptors and by an indirect mechanism involving the activation of PBR (Schlichter et al., 2000). Electrophysiological data have shown that PK11195, an antagonist of PBR (Benavides et al., 1983), reversibly blocked about 60% of the membrane current induced by etifoxine at micromolar concentrations whereas flumazenil, an antagonist of central-type benzodiazepine sites at $GABA_A$ receptors (Sigel and Buhr, 1997), did not affect the etifoxine-induced membrane current (Schlichter et al., 2000).

The goal of the present study was two-fold: firstly, to characterize the nature of the in vitro interaction of etifoxine with the PBR binding site in radioligand binding assays by measuring the displacement of bound [^{3}H]PK11195 in membrane preparations of rat brain. An additional experiment was conducted to evaluate the degree of consistency between plasma and brain etifoxine concentrations in rats receiving an anxiolytic-like dose (50 mg/kg) and IC_{50} values observed in vitro. Secondly, the potential involvement of neurosteroids in the mechanisms underlying the anxiolytic-like activity of etifoxine was investigated in vivo by measuring steroid concentrations in brain and plasma of intact and adrenalectomized-castrated (ADX-CX) rats receiving an anxiolytic-like effective dose of etifoxine (Schlichter et al., 2000). The investigation in ADX-CX rats was designed to probe the effects of etifoxine administration on brain steroid levels, independently of the supply of steroids from peripheral endocrine glands. To further evaluate the hypothesis of neurosteroid implication in the anxiety-reducing activity of etifoxine, finasteride, an inhibitor of 5α-reductase that converts progesterone into 5α-reduced metabolites, was used to focus on the role of allopregnanolone in the effect of etifoxine. The Vogel conflict test (Vogel et al., 1971) was employed, in which it is accepted that the ability of a compound to counteract the suppression of licking response for water induced by an electric shock punishment to thirsty rats (anti-conflict effect) constitutes a reliable parameter predictive of anxiolytic-like activity (Millan and Brocco, 2003). Additionally, the rat brain allopregnanolone levels following pretreatment with finasteride were investigated to determine if the anxiolytic activity of etifoxine may be correlated with levels of this neurosteroid.

2. Materials and methods

2.1. Animals

Male adult Wistar rats (Janvier Laboratories; Genêt-St Isle, France) 5–6 weeks of age and weighing 180–220 g at the start of experiments, were housed in groups of five in propylene cages (L435 × W435 × H140 mm) for at least 7 days before study initiation. The housing facility was temperature (22 ± 2 °C) and relative humidity (50 ± 20%)-controlled and equipped with artificial illumination (7:00 AM to 7:00 PM, lights on). Intact and sham-operated rats had access to food (SAFE-A04; Epinay; France) and tap water ad libitum. Adrenalectomized and castrated rats were maintained in the same conditions as described above, with the exception of drinking water which was substituted by saline. At 2 weeks before the experiments, every animal was handled daily and administered with saline by oral route in order to minimize stress reactions to manipulation. All procedures were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. In vitro [^{3}H]PK11195 binding assay

Radioligand binding assay was adapted from the methods of Desjardins et al. (1999) and Rao and Butterworth (1997). Intact rats (200 g) were quickly sacrificed by decapitation without anaesthesia. Forebrain tissue was dissected out on ice and homogenized in a 20 fold volume of ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenate was centrifuged at 40,000×g for 20 min at 4 °C, and the pellet was washed twice by re-homogenization in fresh buffer and repeated centrifugation at 40,000×g for 20 min at 4 °C. The final pellet was suspended in 50 mM Tris–HCl buffer (pH 7.4) and stored in nitrogen liquid until the day of the binding assay. On the day of the assay, the membrane preparation was thawed, washed twice with 50 mM Tris–HCl buffer (pH 7.4), re-homogenized and centrifuged at 40,000×g for 20 min at 4 °C. The final pellet was suspended in Tris–HCl buffer and used for binding assays. Protein content of the membrane preparation was measured by the BCA protein assay kit (Pierce, Rockford, IL, USA). Binding assays were initiated by the addition of the membrane preparation (100 μg of protein equivalent) to a final volume of 250 μl of 50 mM Tris–HCl buffer (pH 7.4) in 1% DMSO, containing [^{3}H]PK11195 at a final concentration of 2 nM in the presence of etifoxine (2–100 μM) for the competition curve or at 12 concentrations (0.15–15 nM) in the presence of 3 μM etifoxine for the saturation curves. In all binding assays, non-specific binding was measured by addition of excess unlabeled PK11195 (2 μM). After incubation at 4 °C for 2 h, the membrane preparations were vacuum filtered through 0.3% polyethylenimine-pretreated GF/B glass microfibre filters (Whatman International Ltd. Maidstone, UK) and rapidly washed with 3 × 5 ml of ice-cold 50 mM Tris–HCl buffer. The radioactivity retained on filters was determined by liquid scintillation spectrometry with a Tri-carb 2100TR (Packard Instruments, Warrenville, RI, USA), using 5 ml of scintillation liquid (Picofluor 15, Packard Bioscience, Groningen, Netherlands).

Specific [^{3}H]PK11195 binding was defined as the difference between total binding and non-specific binding determined in the presence of 2 μM unlabeled PK11195 (specific/total ratio ≈ 79%). For the competition study between [^{3}H]PK11195 and etifoxine, the concentrations of etifoxine that caused 50% inhibition of specific [^{3}H]PK11195 binding (IC_{50} value) and Hill coefficient (n_H) were determined by non-linear regression analysis of the competition curve. These parameters were obtained by Hill equation curve fitting. For the saturation binding experiments, Scatchard analysis was performed to determine the radioligand equilibrium dissociation constant (K_d) and the binding site density (B_{max}) by computer-assisted non-linear regression of the Scatchard data (Sigma plot v9.0, SPSS Inc.). All values were given as the mean ± standard error of mean (S.E.M.) of three experiments, each performed in triplicate. Mean values were compared using Student's paired two-tailed *t*-test. All statistics were evaluated at a significance level of 5% (SigmaStat. v3.0, SPSS Inc.).

2.3. Plasma and brain etifoxine determination

Separate groups of animals were injected by intraperitoneal route (ip) with etifoxine at doses of 25 or 50 mg/kg using a volume of 5 ml/kg. A previous study showed that these doses produced an anxiolytic-like effect in rats (Schlichter et al., 2000). Animals were sacrificed by decapitation at 0.25, 0.5 and 1 h after injection and blood was collected in propylene tubes containing lithium heparinate. After centrifugation (2000×*g* for 3 min at room temperature), plasma samples were stored at −20 °C for etifoxine determination. The brain (without cerebellum) was quickly removed from the skull and stored also at −20 °C until assayed. Etifoxine concentrations in plasma and brain were measured using a liquid chromatography tandem mass spectrometric method LC/MS/MS. Briefly, 50 µl of plasma or 100 mg of brain, 25 µl of internal standard solution (AB2446, Biocodex, France; 2 µg/ml) and 1 ml of 0.1 M KH_2PO_4 (pH9) were mixed, followed by the addition of 5 ml of 95/5 v/v hexane/propanol-2. Samples were vortexed and frozen at −70 °C for 10 min. The organic layer was transferred and evaporated to dryness under nitrogen at 45 °C. Residues from plasma and brain samples were reconstituted with 1 ml and 500 µl, respectively of acetonitrile/ammonium acetate 10 mM (0.1%NH_4OH) (50/50 v/v). High-pressure liquid chromatography separation was achieved using a XTERA MC (Waters) C18 column (2.1 × 50 mm, 3.5 µm) maintained at 30 °C and at a flow rate of 200 µl/min. Detection was performed in positive, multiple reaction monitoring mode using a Quattro LCZ (Micromass Inc. Palo Alto, CA, USA) with an electron impact source as the LC/MS/MS interface. The limits of quantification were set at 10 ng/ml and 20 ng/g for the plasma and the brain, respectively. The intra- and inter-assay coefficients of variation ranged between 1.3% and 4% and between 1.2% and 2.6%, respectively. The levels of etifoxine in plasma (ng/ml) and in brain (ng/g) were expressed as total drug concentration.

2.4. Measurements of steroid concentrations

Separate experiments were performed in adrenalectomized-castrated or sham-operated rats 15 days after surgery. Etifoxine at 50 mg/kg dose or its vehicle were administered intraperitoneally using a volume of 5 ml/kg and animals were sacrificed by rapid decapitation without anaesthesia 30 min later. Steroid analysis was carried out in individual samples from brain and plasma as previously described (Liere et al., 2000) with slight modifications. Briefly, steroids were extracted from 300 mg and 1 ml of rat brain and plasma, respectively, with 10 volumes of methanol (MeOH). Appropriate internal standards were added, i.e. [1,2,4,5,6,7-^{2}H]-5α-dihydroprogesterone (2 ng) for 5α-dihydroprogesterone (5α-DHP), 5β-androstane-3β-ol-17-one (1 ng) for pregnenolone, progesterone and allopregnanolone, and [9,12,12-^{2}H]-cortisol (100 ng) for corticosterone. Pregnenolone, progesterone and allopregnanolone were kind gifts from Roussel-Uclaf (Romainville, France), 5α-dihydroprogesterone, corticosterone, 5β-androstane-3β-ol-17-one were purchased from Sigma-Aldrich (St. Louis, MO, USA) and $^2H_3$-cortisol was obtained from CIL Cambridge Isotope Laboratories Inc. (Andover, MA, USA). The unconjugated steroid fraction was eluted with 5 ml MeOH/H_2O (85/15, v/v) by solid phase extraction (SPE) on C18 silica minicolumns (500 mg, International Sorbent Technology, Mid Glamorgan, UK). Steroids were then separated by high performance liquid chromatography using a system from Thermoelectron (San Jose, CA, USA), consisting of a P1000XR quaternary pump and an AS 100 XR TSP autoinjector. HPLC was achieved with a Lichrosorb Diol column (25 cm × 4.6 mm, 5 µm) at 30 °C. Steroid elution was performed at a flow rate of 1 ml/min, with a solvent system composed of hexane and mixture A (90/10, v/v), the latter being composed of hexane–isopropanol (85/15, v/v). HPLC was coupled to a fraction collector (2002 model, Gilson). Three fractions were obtained containing 5α-dihydroprogesterone in the first, progesterone, allopregnanolone and pregnenolone in the second, and corticosterone in the third. Each steroid fraction was derivatized with either a mixture of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide/ammonium iodure/dithioerythritol (1000/2/5, v/v) for the first fraction or heptafluorobutyric anhydride for the others. Steroids were then identified and quantified by gas chromatography (GC)/mass spectrometry (MS). GC was performed in the splitless mode with a GC 8000 Top gas chromatograph (Carlo Erba) and the oven temperature was ramped up from 50 °C to 330 °C. The mass spectrometer (model 150, Finnigan Automass, Argenteuil, France) was operated in the electron impact mode and quantification was done in the single-ion monitoring mode. The ionization energy and ionization chamber temperature were 70 eV and 180 °C, respectively. Measurements for each brain or plasma sample were made in duplicate. The intra- and inter-assay coefficients of variation were roughly 5–9% and 13–17%, respectively. The detection limits for endogenous pregnenolone, progesterone, 5α-DHP, allopregnanolone and corticosterone were, respectively, 0.15; 0.05; 0.3; 0.15 and 1.5 ng/g using 300 mg of brain tissue, and 0.05; 0.02; 0.10; 0.15 and 0.5 ng/ml with 1 ml of plasma. Data were expressed as mean ± S.E.M. Statistical analysis of data was performed using two-tailed Student's *t*-test. The requirement for statistical significance was set at $P < 0.05$ (SigmaStat. v3.0, SPSS Inc.).

2.5. Water-lick conflict test

The procedure was modified from a method described previously (Vogel et al., 1971). Rats were water-deprived for

48 h prior to the test session. Each animal was then placed in a conflict test box (Letica-model 8600-Spain) for 5 min and was allowed to explore and to lick 40 times the drinking spout before being removed from the box. Only rats licking during this session were used. Six hours after this adaptation session, the second experimental session, lasting 3 min started automatically when the rat completed 20 licks and received the first mild electric shock (0.6 mA, 1 s). After 20 unpunished licks, subsequent licking was punished. The number of shocks accepted throughout the 3 min experimental session was recorded. Etifoxine (12.5–50 mg/kg) progesterone (12.5–50 mg/kg) and clonazepam (0.025–0.1 mg/kg) were ip administered under a volume of 5 ml/kg 30 min prior to the experimental session. Progesterone was used as a positive control because it is known that its activity is mediated by the neuroactive steroid allopregnanolone (Bitran et al., 1991; Reddy et al., 2004). Clonazepam, whose activity does not implicate allopregnanolone (Anholt, 1986), was used as a negative control. Finasteride was subcutaneously (sc) administered at a 50 mg/kg dose under a volume of 2 ml/kg 4 h and 1.5 h before the experimental session. The chosen dose of finasteride was based on previous behavioral and biochemical studies in rodents showing that 50 mg/kg finasteride decreased 5α-reductase enzyme activity by 60–80% (Lephart et al., 1996; Van Doren et al., 2000). Data are expressed as mean ± S.E.M. When the compounds were administered alone, statistical analysis was made using the Kruskal–Wallis test followed by the post hoc Dunn's test with the control group as reference. When the compounds were co-administered with finasteride, statistical analysis used the two-way analysis of variance (ANOVA) with the compounds and finasteride as factors, followed by the Student–Newman–Keuls test (SNK procedure) to locate the differences between the experimental groups. The accepted level of significance was $P < 0.05$ (SigmaStat. v3.0, SPSS Inc.).

2.6. Effects of finasteride on the etifoxine-induced allopregnanolone level changes in the rat brain

To assess the effect of brain allopregnanolone content changes on etifoxine's activity, an additional experiment was performed. Separate groups of rats were treated with finasteride (50 mg/kg) associated or not with etifoxine at a 50 mg/kg dose. The finasteride administrations followed the same schedule as described above. The procedure for the brain allopregnanolone levels determination was identical to that described above. The statistical analysis of data used the two-way ANOVA with finasteride and etifoxine as factors followed post hoc by the SNK procedure to compare the individual means. A value of $P < 0.05$ was considered to be statistically significant in all the cases (SigmaStat. v3.0, SPSS Inc.).

2.7. Drugs

For binding assays, stock solutions (100 mM) etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4*H*-3,1-benzoxazine hydrochloride; FW = 337.25; batch 196, Biocodex, France) were made up in dimethyl sulfoxide (DMSO) and diluted before use in distilled water (final concentration of DMSO < 0.1%). [^{3}H]PK11195 (specific activity, 83.5 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA, USA) and non-radioactive PK11195 from Tocris (Bristol, UK). For in vivo studies (steroid and etifoxine levels determination and conflict test), etifoxine (batches 114, 196 and 219) was suspended in saline solution (0.9% NaCl) containing 1% Tween 80 (v/v) whereas clonazepam (Rivotril®) was dissolved in saline solution. Progesterone (Sigma, France) and finasteride (Sigma, France) were suspended in 20% and 40% 2-hydroxypropyl-β-cyclodextrin (Sigma, France) (w/v) in distilled water, respectively. Control animals received an equivalent volume of vehicle solutions.

3. Results

3.1. [^{3}H]PK11195 binding

The specific binding of [^{3}H]PK11195 was inhibited in a concentration-dependent manner by etifoxine ($IC_{50} = 18.3 \pm 1.2$ μM; $n_H = 0.9 \pm 0.3$ which was not significantly different from the value of 1) (Fig. 1a). Scatchard analysis of the [^{3}H]PK11195 binding data (Fig. 1b) revealed that etifoxine at 3 μM significantly decreased the apparent B_{max} value relative to the control (732 ± 79 versus 1352 ± 125 fmol/mg protein for control, $P = 0.014$) without significantly altering the dissociation constant (K_d) (2.6 ± 0.2 nM versus 4.5 ± 0.9 nM for control, $P = 0.116$).

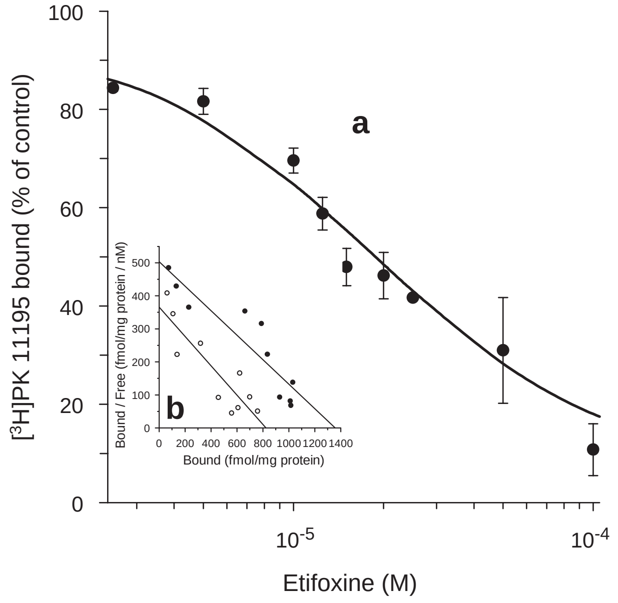

Fig. 1. (a) Concentration-dependent inhibition of [^{3}H]PK11195 specific binding to membranes from rat forebrain by etifoxine. Each point represents the mean ± S.E.M. of three experiments carried out in triplicate. (b) Scatchard analysis of specific [^{3}H]PK11195 binding to membranes from rat brain in the absence (full circles) or presence (open circles) of 3 μM etifoxine (EFX). The data are from a single experiment performed in triplicate and are representative of three experiments with similar results.

Table 1
Plasma and brain etifoxine concentrations after ip administration of two doses in rats

Time after injection (h)	Plasma (ng/ml)		Brain (ng/g)	
	25 mg/kg	50 mg/kg	25 mg/kg	50 mg/kg
0.25	2104±437	5638±925	4348±909	11404±1940
0.5	1134±177	3725±686	2109±531	7830±1466
1	1011±246	2761±165	1739±368	5186±494

Values represent the mean±S.E.M. of 6 animals.

3.2. Plasma and brain etifoxine determination

In the time period between 0.25 and 0.5 h after an ip injection of 25 or 50 mg/kg dose, the range of mean plasma concentrations of etifoxine varied between 1134 and 5638 ng/ml, corresponding to a concentration range of 3–17 μM (Table 1). After the same treatments, the corresponding mean brain concentrations of etifoxine ranged between 2109 and 11404 ng/g. These values correspond approximately to a concentration range of 9–48 μM.

3.3. Effects of etifoxine on steroid concentrations

The intraperitoneal administration of etifoxine (50 mg/kg) in sham-operated rats resulted, after 30 min, in significant increases in the brain concentrations of pregnenolone ($P<0.001$), progesterone ($P<0.01$), 5α-dihydroprogesterone ($P<0.01$) and allopregnanolone ($P<0.01$) compared with the control (Table 2). In this group, etifoxine also enhanced the plasma concentrations of these steroids ($P<0.05$), except for 5α-dihydroprogesterone levels which were undetectable as in the controls. In ADX-CX animals, increased brain levels of pregnenolone ($P<0.001$), progesterone ($P<0.01$), and allopregnanolone ($P<0.01$), but not 5α-dihydroprogesterone, were also observed following etifoxine injection, while in the plasma of those animals, concentrations of pregnenolone were not affected by the drug and the levels of the other steroids were not detectable. With regard to corticosterone, no significant differences were noted between control and etifoxine-treated sham-operated rats, in both plasma and brain, and the steroid was undetectable in those tissues in ADX-CX rats.

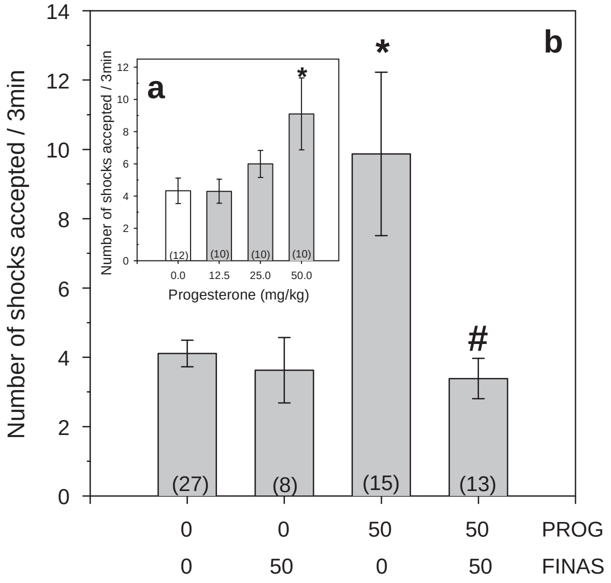

Fig. 2. (a) Effects of different doses of progesterone (PROG) administered alone on the number of shocks accepted during a 3 min session. (b) Effects of finasteride (FINAS) on progesterone-induced increase in number of shocks accepted during a 3 min session. Finasteride was administered 50 mg/kg, sc, 4 and 1.5 h before the session and progesterone (50 mg/kg) was administered ip 30 min before the session. Values are expressed as means±S.E.M. of the number of animals indicated inside each column. *$P<0.05$ versus vehicle-treated group. $^{\#}P<0.05$ versus progesterone-treated group.

3.4. Vogel's conflict test

When the compounds were administered alone (Figs. 2a, 3a and 4a), significant increases in punished licking were noted following the injection of progesterone (50 mg/kg), etifoxine

Table 2
Effects of etifoxine (EFX) on steroid concentrations in the brain and plasma of sham or ADX-CX rats

	Concentration in brain (ng/g) or plasma (ng/ml)				
	Pregnenolone	Progesterone	5α-DHP	Allopregnanolone	Corticosterone
Sham					
Brain					
Control	1.56±0.24	0.35±0.06	0.94±0.19	0.33±0.12	13.46±1.72
EFX	6.53±0.73*	1.28±0.22*	1.99±0.19*	0.72±0.07*	12.69±1.24
Plasma					
Control	1.03±0.14	0.23±0.07	nd	0.12±0.05	20.09±4.68
EFX	4.35±0.65*	0.57±0.12*	nd	0.41±0.04*	23.88±3.22
ADX-CX					
Brain					
Control	0.54±0.05	0.17±0.02	nd	0.16±0.04	nd
EFX	1.12±0.04*	0.26±0.01*	nd	0.36±0.04*	nd
Plasma					
Control	0.08±0.01	nd	nd	nd	nd
EFX	0.11±0.02	nd	nd	nd	nd

Rats were sacrificed 30 min after the administration of EFX (50 mg/kg, ip). Data are mean±S.E.M. ($n=8$).
5αDHP, 5α-dihydroprogesterone; nd=not detectable. *$P<0.05$ versus respective vehicle-treated group.

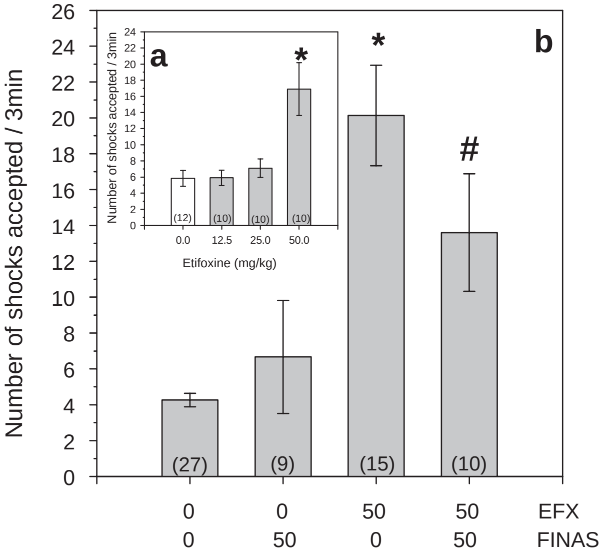

Fig. 3. (a) Effects of different doses of etifoxine (EFX) administered alone on the number of shocks accepted during a 3 min session. (b) Effects of finasteride (FINAS) on etifoxine-induced increase in number of shocks accepted during a 3 min session. Finasteride was administered 50 mg/kg, sc, 4 and 1.5 h before the session and etifoxine (50 mg/kg) was administered ip 30 min before the session. Values are expressed as means ± S.E.M. of the number of animals indicated inside each column. *$P<0.05$ versus vehicle-treated group. #$P<0.05$ versus etifoxine-treated group.

(50 mg/kg) and clonazepam (0.1 mg/kg) as compared with the vehicle-control groups. These effects were consistent with anxiolysis. All compounds failed to produce any significant effect on spontaneous water drinking or shock thresholds even at the highest doses tested which therefore had no analgesic effect (data not shown). In the case of combination of finasteride with progesterone (Fig. 2b), the two-way ANOVA showed a significant progesterone effect [$F(1,59)=4.28$; $P=0.04$], a significant finasteride effect [$F(1,59)=6.83$, $P=0.01$] and a significant progesterone × finasteride interaction [$F(1,59)=5.06$, $P=0.03$] for the number of accepted shocks. Post-hoc test (SNK procedure) showed that progesterone at 50 mg/kg dose enhanced the number of shocks accepted ($P<0.001$) as compared with the controls and that finasteride was ineffective on its own ($P=0.803$) but blocked the effect of progesterone ($P<0.001$). After a finasteride pretreatment with etifoxine (Fig. 3b), the two-way ANOVA showed a significant effect of etifoxine [$F(1,57)=27.45$, $P<0.001$], a non-significant finasteride effect [$F(1,57)=0.90$, $P=0.35$] and a significant etifoxine × finasteride interaction [$F(1,57)=4.22$, $P=0.04$] for the number of shocks accepted. Further analysis (SNK procedure) showed that etifoxine at 50 mg/kg dose increased the number of shocks received ($P<0.001$) as compared with the controls and that finasteride at the 50 mg/kg ineffective dose ($P=0.42$) attenuated significantly the effects of etifoxine ($P=0.04$). When clonazepam was co-administered with finasteride (Fig. 4b), the two-way ANOVA revealed a significant effect of clonazepam [$F(1,62)=22.60$, $P<0.001$] but a not significant effect of finasteride [$F(1,62)=0.18$, $P=0.68$] and a non-significant clonazepam × finasteride interaction [$F(1,62)=0.11$, $P=0.75$] for the number of shocks accepted. Further post-hoc tests revealed that clonazepam at 0.1 mg/kg increased significantly the number of shocks received ($P<0.001$, SNK procedure) and this effect was not reversed ($P=0.59$, SNK procedure) by finasteride. As previously observed, finasteride alone had no significant effect on the number of shocks accepted ($P=0.949$, SNK procedure) as compared with the controls.

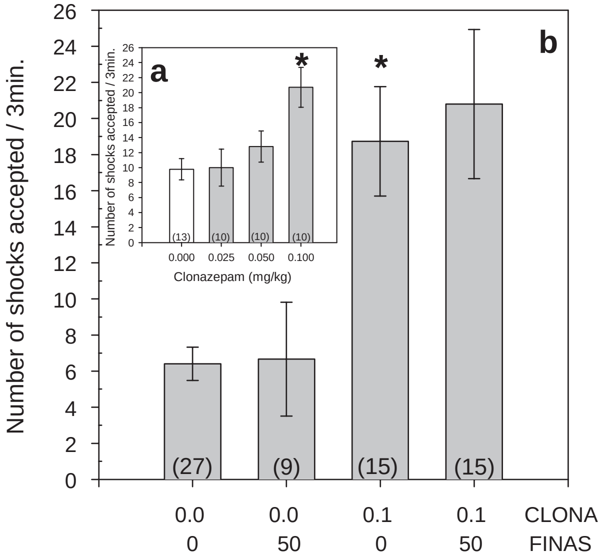

Fig. 4. (a) Effects of different doses of clonazepam (CLONA) administered alone on the number of shocks accepted during a 3 min session. (b) Effects of finasteride (FINAS) on clonazepam-induced increase in number of shocks accepted during a 3 min session. Finasteride was administered 50 mg/kg, sc, 4 and 1.5 h before the session and clonazepam (0.1 mg/kg) was administered ip 30 min before the session. Values are expressed as means ± S.E.M. of the number of animals indicated inside each column. *$P<0.05$ versus vehicle-treated group.

3.5. Effects of finasteride on the etifoxine-induced changes in allopregnanolone brain level

The two-way ANOVA revealed significant main effects of finasteride pretreatment [$F(1,26)=54.17$, $P<0.001$] and a not significant main effect of EFX [$F(1,26)=3.28$, $P=0.082$] for the brain allopregnanolone levels (Table 3). A significant finasteride × etifoxine interaction for brain allopregnanolone level was found [$F(1,26)=9.25$, $P=0.005$]. Post hoc comparisons (SNK procedure) showed that etifoxine produced a significant 1.8-fold increase in allopregnanolone level as

Table 3
Effects of finasteride and etifoxine on brain allopregnanolone levels in the rat

Treatment	*n*	Brain allopregnanolone (ng/g)
Control	7	0.81 ± 0.26
Finasteride	8	0.22 ± 0.04*
EFX	7	1.47 ± 0.13*
Finasteride + EFX	8	0.05 ± 0.01#

Finasteride was administered 50 mg/kg, sc, 4 and 1.5 h before sacrificing and etifoxine (EFX, 50 mg/kg-ip) was administered 30 min before sacrificing.
Data are mean ± S.E.M. n = number of animals used.
*$P<0.05$ versus vehicle-treated group; #$P<0.05$ versus EFX-treated group.

compared with the controls ($P = 0.003$). Pretreatment with finasteride completely abolished etifoxine-induced elevation of allopregnanolone level ($P < 0.001$). As reported by other investigators (Lephart et al., 1996; Van Doren et al., 2000), finasteride, administered alone, significantly reduced the allopregnanolone levels as compared with the controls ($P = 0.005$).

4. Discussion

Our observation that etifoxine uncompetitively inhibited the binding of [^{3}H]PK11195 to PBR in the brain suggests that the compound binds with a micromolar affinity to this receptor. While this value appears higher than the submicromolar affinities exhibited by other ligands of PBR (Le Fur et al., 1983), the present study has also shown that plasma and brain concentrations of etifoxine within the micromolar range are observed following an ip administration of doses with anxiolytic-like effects in rat. Based on these observations, it is realistic to assume that etifoxine reaches intracellular concentrations in the micromolar range, thus providing a relevant in vitro–in vivo correlation. Etifoxine probably acts at a site on the PBR that is not identical with those that bind [^{3}H]PK11195. The PBR, originally localized in peripheral tissues (adrenals, heart, kidney, testis), has been found in the brain mainly in glial cells (Schoemaker et al., 1981; Benavides et al., 1983; Verma and Snyder, 1989; Gavish et al., 1999). Many functions have been attributed to PBR, including a role in steroidogenesis (Papadopoulos et al., 1990), calcium flow (Cantor et al., 1984) and cellular respiration (Hirsch et al., 1989). In some biological processes associated with an acute stress, the PBR was highly activated (up-regulation of PBR density) in peripheral organs (heart, adrenals) and in brain (Basile et al., 1987; Drugan and Holmes, 1991; Gavish et al., 1999). The PBR is located mainly on the mitochondrial membrane and also on the plasma membrane (Woods and Williams, 1996; Casellas et al., 2002) and, in contrast to the central benzodiazepine receptor, it is not coupled to the GABA$_A$ receptor (Anholt, 1986). Biochemical, pharmacological and molecular studies have demonstrated that the PBR is a five transmembrane domain mitochondrial protein involved in the regulation of cholesterol transport (see review by Lacapère and Papadopoulos, 2003). The activation of the PBR inducing stimulation of steroid production has been extensively studied (Papadopoulos, 1993). Steroidogenesis begins with the translocation of cholesterol from the outer to the inner mitochondrial membrane (Papadopoulos, 1993) and its conversion to pregnenolone, the first synthesized neuroactive steroid (Mellon and Griffin, 2002). Pregnenolone is then metabolized to steroid intermediates like progesterone and its 3α,5α-reduced metabolite, allopregnanolone.

In the present study, measurements of steroid levels in the brain and plasma of ADX-CX versus sham rats strongly suggest that etifoxine increases the concentrations of several neurosteroids in the brain, independently of the peripheral endocrine sources (testis and adrenal glands). Indeed, in ADX-CX rats, etifoxine increased brain concentrations of PREG significantly (two-fold) while corresponding plasma concentrations remained unchanged. Similarly, in these animals, progesterone and allopregnanolone were undetectable in the plasma, whereas they were still found present in the brain. The fact that 5α-dihydroprogesterone, the precursor of allopregnanolone, was not detectable in the brain of ADX-CX rats may be explained by the limited sensitivity of the assay for this steroid (i.e. 100 pg taking in account the entire analytical protocol including GC-MS sensitivity, dilution factor and experimental losses) as well as its very low endogenous levels. Overall, our results demonstrated that the anxiolytic compound etifoxine is able to significantly increase a number of neuroactive neurosteroids including pregnenolone, progesterone and allopregnanolone. The significant increases in brain neurosteroid content by etifoxine is probably associated with an increase in their local synthesis and/or in their disappearance rate (release, transport or degradation).

Interestingly, under the present conditions, acute etifoxine administration increased the neuroactive steroid allopregnanolone in rat brain to concentrations (0.7–1.5 ng/g or 3–5 nM) known to interact with the GABA$_A$ receptor in vitro (Majewska et al., 1986; Morrow et al., 1987). Among the known endogenous positive allosteric modulators of GABA$_A$ receptors, allopregnanolone is one of the most potent and efficacious steroids (Majewska et al., 1986; Lambert et al., 1999; Compagnone and Mellon, 2000) and this regulatory mechanism is believed to underlie many of its pharmacological effects on animal behavior, including anxiety (Akwa and Baulieu, 1999; Bitran et al., 2000). The hypothesis that etifoxine-induced elevations in the anxiolytic steroid allopregnanolone contribute to the anxiolytic-like effect of this compound is strengthened by the present results obtained in the presence of finasteride, known to block the biosynthesis of 5α-reduced metabolites from progesterone, including allopregnanolone (see Rupprecht, 2003). Our results on anxiety-like behavior are in line with outcomes of studies in the literature showing that finasteride blocked the anticonvulsant and anxiolytic effects of progesterone thus supporting the involvement of the 5α-reduced metabolites in the pharmacological and the behavioral activity of progesterone (Bitran et al., 1991; Kokate et al., 1999; Reddy et al., 2005). Conversely, the anti-conflict effect of clonazepam, described to bind with high affinity to most GABA$_A$ receptors but not to the PBR (Anholt, 1986), was not reversed by finasteride in the present study. Although finasteride reduced the brain allopregnanolone levels by inhibiting the conversion of progesterone to allopregnanolone (Lephart et al., 1996; Van Doren et al., 2000) with a concomitant decrease in allopregnanolone levels as shown by the present study in the rat brain, it did not have behavioral effects in the controls. In fact, endogenous levels of allopregnanolone in naïve rats are very low, certainly below physiologically relevant concentrations (Purdy et al., 1991). It is noteworthy that the anti-conflict effect of etifoxine was significantly attenuated, and not completely reversed by finasteride pretreatment as shown with progesterone. These findings suggest that other well-described mechanisms of action of etifoxine, such as the facilitation of the GABAergic transmission by a direct positive allosteric effect on GABA$_A$

receptors (Schlichter et al., 2000; Verleye et al., 2002), may partially underlie its anxiolytic-like activity. This hypothesis is strengthened by the fact that the anxiolytic effect of etifoxine is not completely suppressed even though the brain allopregnanolone levels were drastically reduced in the presence of finasteride. In addition, it is possible that the brain allopregnanolone concentrations (0.7–1.5 ng/g or 3–5 nM) achieved after etifoxine treatment are not fully relevant to the anxiolytic behavior. Actually, it has been shown that the anxiolytic activity of allopregnanolone was associated with brain concentrations ranging between 3 and 6 ng/g (10–20 nM) (Bitran et al., 1993; Marx et al., 2000). Overall, the anxiolytic-like properties of etifoxine could be mediated at least in part by the stimulation of the production of neurosteroids after binding to the PBR coupled with the potentiation of $GABA_A$ receptor function by allopregnanolone produced in the brain. A delineation of the respective magnitudes of the two mechanisms of action of etifoxine contributing to its anxiolytic properties would warrant further investigation. Interestingly, recent studies have revealed a relationship between alterations in cerebrospinal fluid or plasma levels of certain neuroactive steroids and particular types of human anxiety disorders, i.e. decreased levels of allopregnanolone in subjects with anxiety associated with major unipolar depression or with alcohol withdrawal (Uzunova et al., 1998; Romeo et al., 2000) and increased levels of pregnenolone, progesterone and allopregnanolone in panic disorders (Brambilla et al., 2003; Strohle et al., 2002). Recent preclinical and clinical studies have shown that a selective serotonin-uptake inhibitor like fluoxetine and an atypical antipsychotic drug like olanzapine enhance the synthesis of allopregnanolone in the brain contributing in part to the improvement of anxio-depressive symptomology (Uzunov et al., 1996; Frye and Seliga, 2003). Neuroactive neurosteroids have been implicated in pathophysiological mechanisms underlying a variety of emotional and affective disorders such as anxiety and depression (Pisu and Serra, 2004) and enhancement of their roles or endogenous concentrations by drugs such as etifoxine may open novel therapeutic avenues.

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