Lack of interaction between etifoxine and CRF$_1$ and CRF$_2$ receptors in rodents

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Abstract

Hyperactivity of the corticotropin-releasing factor (CRF) system occurs in some patients with anxiety disorders and depression. Blockade of CRF$_1$ and CRF$_2$ receptors can underlie the anxiolytic effects of drugs. In the present investigation, in vivo and in vitro studies were designed to determine whether the anxiolytic drug etifoxine, known to enhance GABAergic synaptic transmission, behaves also as a CRF$_1$ and CRF$_2$ receptor antagonist. A drug exerting multiple actions may be of clinical interest in the treatment of various different forms of mood disorders. Using two animal models, it was found that etifoxine reversed the excess CRF-induced grooming but not the hypo-locomotion of the rat placed in an open field. Etifoxine attenuated the CRF-induced gastric emptying delay in the mouse. On the other hand, in vitro, binding of etifoxine to CRF$_1$ and CRF$_2$ receptors on rat brain membranes was negligible and functionally, etifoxine did not block the CRF$_1$ and CRF$_2$ activation-induced cAMP production in presence of CRF in human neuroblastoma SH-SY5Y cells. The selective anxiolytic properties of etifoxine appear unrelated to an antagonist activity at the CRF$_1$ and CRF$_2$ receptors. The decrease in CRF activity produced by etifoxine may be related to its GABAergic properties.

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1. Introduction

The corticotropin-releasing hormone, is a key mediator of mammalian endocrine, behavioral, autonomic, and immune responses to stress (Vale et al., 1981; Owens and Nemeroff, 1991; Bale and Vale, 2004). Within the hypothalamic–pituitary–adrenal axis, CRF is the principal regulator of pituitary adrenocorticotropic hormone (ACTH) and adrenal glucocorticoid secretion in response to stressful stimuli. Basic and clinical research studies indicate that elevated central CRF levels are involved in the etiology of stress-related psychiatric, physiological and behavioral disorders like depression and anxiety (Holsboer et al., 1984; Nemeroff et al., 1984; Linthorst et al., 1997). In the rat and human brain, CRF acts at two distinct G-protein coupled receptors, CRF$_1$ and CRF$_2$, each having a distinct anatomical localization and pharmacology (Perrin and Vale, 1999; Dautzenberg and Hauger, 2002). Recent studies have been conducted to establish the respective roles of CRF$_1$ and CRF$_2$ in stress-related physiological and behavioral processes (Risbrough et al., 2004; De Groote et al., 2005). Although there is strong evidence that CRF$_1$ is intimately involved in anxiety-related behavior (Ayala et al., 2004), a role for CRF$_2$ cannot be excluded and according to some, seems to be underestimated (Takahashi, 2002). Some evidence indicates that anxiety behavior mediated by CRF$_1$ activation is reduced when CRF$_2$ is stimulated (Bale et al., 2002; Valdez et al., 2002). Nevertheless, other evidence indicates that CRF$_2$ is also capable of inducing anxiety-like behavior (Pellemounter et al., 2002). These findings have led to the hypothesis that the CRF$_1$ and CRF$_2$ receptors could become novel therapeutic targets and that CRF$_1$ and CRF$_2$ antagonists could be evaluated in the treatment of anxiety disorders associated with a maladaptive stress response (Nemeroff, 1998; Holmes et al., 2003). One approach to testing that hypothesis is to determine whether clinically established anxiolytic compounds behave as CRF$_1$ and CRF$_2$ antagonists. This approach was used in the present study with...
etifoxine as the test compound. Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride, Stresam\textsuperscript{6}), a molecule structurally unrelated to benzodiazepines has demonstrated anxiolytic properties in rodents (Boissier et al., 1972; Verleye and Gillardin, 2004) and in humans in the treatment of adjustment disorder with anxiety in humans (Servant et al., 1998), a disorder described in Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, Fourth ed., 1995). In animals, anxiolytic-like properties of etifoxine, at doses ranging between 12.5 and 50 mg/kg by intraperitoneal route, were evidenced in classical anxiety tests, e.g. the Vogel’s conflict test (Schlichter et al., 2000) and in the stress-induced hyperthermia paradigm in rats (Verleye and Gillardin, 2004).

We have conducted a number of studies on the mechanism of action of etifoxine showing an enhancement of GABAergic synaptic transmission (Verleye et al., 1999, 2002; Schlichter et al., 2000). Recent binding and electrophysiological experiments have demonstrated that etifoxine binds to \( \gamma \)-amino butyric acid\( \alpha \) (GABA\( \alpha \)) receptors via an allosteric site near or on the chloride channel, which differs from that of benzodiazepines (Verleye et al., 1999, 2002). Recently, it was been shown that the etifoxine enhancing effects of the GABAergic synaptic transmission are mediated by the \( \beta_2 \) and \( \beta_3 \) subunits composing the GABA\( \alpha \) receptor (Hamon et al., 2003). However, it is not known whether this anxiolytic interacts directly with the CRF\( _1 \) and CRF\( _2 \) receptors. The pathophysiology of anxiety disorders is complex: it is linked to abnormal regulation of several neurobiological components including GABA, monoamines and the CRF system. Although effective treatments exist, there is still considerable room for improvement. An anti-anxiety drug with multiple actions could be beneficial in the treatment of different pathological anxious states involving more than a single neurotransmitter system dysfunction (Bourin and Lambert, 2002; see review by Millan, 2003).

In the present study, the hypothesis that etifoxine might behave as a CRF\( _1 \) and CRF\( _2 \) antagonist was evaluated using in vivo and in vitro approaches. In vivo studies were conducted in rat and mouse models based on the fact that central injections of CRF reproduce the patterns of behavioral and autonomic changes induced by stress (Williams et al., 1987; Habib et al., 2000). In the rat, CRF induces an increase in grooming associated with a decrease of exploration when the animal is exposed to an unfamiliar environment (Dunn and Berridge, 1990; Koob, 1999). In the mouse, central administration of CRF induces stress-related disturbances in gastrointestinal motor function such as an inhibition of gastric emptying of a solid meal (Sheldon et al., 1990; Martinez et al., 2002; Zorrilla et al., 2003). In vitro binding studies explored the interactions between etifoxine and the CRF\( _1 \) and CRF\( _2 \) receptors in rat brain membrane preparations. The functional counterpart of this binding was studied concurrently in vitro by testing the effects of etifoxine on CRF-induced stimulation of cAMP in human neuroblastoma SH-SY5Y cells. The CRF receptor antagonist, \( \alpha \)-helical CRF\( _{9-41} \) (\( \alpha \)-h-CRF), which binds to both subtypes of CRF receptors (Rivier et al., 1984), was used for comparison.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats (220–260 g, 6–7-week old; Janvier breeding, France) were used for the open field test and male NMRI mice (25–30 g, 6–7-week old; Janvier breeding, France) for the gastric emptying test. Male Sprague–Dawley rats (175–200 g, 5–6-week old; Charles River, France) were used for receptor binding assays. All the animals were allowed to habituate to the handling and to the laboratory environment for at least 1 week before any experimental manipulation. Rats and mice were housed in groups of 5 and 10 in (43 cm \( \times \) 43 cm \( \times \) 19 cm) polycarbonate cage, respectively. Animals had unrestricted access to food (A04-Safe, France) and tap water and were maintained under a 12-h-light/12-h-dark cycle (lights on at 7:00 a.m.,) with temperature at 20–24\(^\circ\)C and relative humidity at 30–70\%. All experiments were performed between 9:00 a.m. and 4:00 p.m. All procedures with animals were in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.2. Drugs

For the in vivo tests (open field, gastric emptying), CRF (rat and human CRF; Tocris, France) and \( \alpha \)-h-CRF (Tocris, France) were dissolved in sterile saline (0.9\% NaCl in sterile distilled water) immediately before use. Etifoxine (Biocodex, France) was suspended in a saline solution of 1\% Tween 80 (v/v). Control animals received the same volume of appropriate vehicles (etifoxine vehicle or \( \alpha \)-h-CRF vehicle and CRF vehicle). For the binding assay, stock solutions (100 nM) of etifoxine were made up in pure dimethylsulfoxide (DMSO) and diluted before use in distilled water (final concentration of DMSO < 1\%). CRF (rat and human CRF; Tocris, France) was dissolved in 1\% DMSO. \(^{125}\)I iodohistidyl 32 human CRF (\(^{125}\)I-h-CRF; IM 189; specific activity, 2000 Ci/mmol) was purchased from Amersham Biosciences (France). For cAMP levels measurements, stock solutions (100 mM) of etifoxine were made up in pure DMSO and diluted before use in distilled water (final concentration of DMSO < 0.1\%). The peptides CRF (rat and human CRF) and \( \alpha \)-h-CRF were purchased from Bachem (Switzerland) and Sigma (France), respectively, and dissolved in distilled water. \(^{125}\)I cAMP (specific activity, 2200 Ci/mmol) was obtained from Perkin-Elmer (France).

2.3. i.c.v. injection of CRF in the rodent

2.3.1. Open field test in the rat

Rats were anesthetized (80 mg/kg ketamine and 10 mg/kg xylazine, i.p.) and were placed on a Kopf stereotaxic apparatus. A 23 gauge stainless-steel guide cannula was lowered 1 mm above the left lateral ventricle and anchored to the skull with screws and dental cement. With the tooth bar positioned 3.3 mm below interaural zero, the coordinates were: anterior/posterior –0.8 mm, medial/lateral ±1.5 mm relative to bregma, and 3.6 mm below the skull surface at the point of entry (Paxinos and Watson, 1986). Following surgery, animals were housed individually and were given extencillin (50,000 IU/0.3 ml/rat, intramuscularly) to minimize infection risk. Rats were allowed 1 week to recover before testing. At the end of each experiment, correct placement of the guide cannula was verified by injection of methylene blue (1% in distilled water, v/v) and brain section. Only animals with verification of dye in all ventricles were included in the data analysis.

CRF and \( \alpha \)-h-CRF or vehicle were intracerebroventricularly (i.c.v.) micro-injected in 5\( \mu \)l over 1 min by using a Hamilton micro syringe with a 30-gauge stainless-steel needle projecting 1 mm past the end of the guide cannula and attached to polyethylene 10 and 50 tubing. The injection cannula was left in place for a further 1 min to prevent efflux. Etifoxine or its vehicle (5 ml/kg) was intraperitonealy (i.p.) administered 25 min prior the CRF injection while \( \alpha \)-h-CRF or its vehicle was i.c.v. infused 5 min before CRF injection. The animals were subjected to the open field test 5 min after the CRF infusion. A dose of 10\( \mu \)g of CRF was selected on the basis of pilot dose–effect studies in our laboratory and from literature data (Morley and Levine, 1982).

The open field consisted of a walled white arena (100 cm \( \times \) 100 cm \( \times \) 50 cm). The floor was divided into 25 identical sectors (20 cm \( \times \) 20 cm) cm brightly illuminated by 4–50 W bulbs (940 lx) situated 2 m above the field.
Each animal was placed individually in the center of the apparatus and its behavioral parameters were recorded for 5 min. Locomotion was scored by the number of floor sections entered and a chronometer was used to measure grooming duration (s) or total time the animals touched the head and/or the body with the forepaws. The open field apparatus was washed with a 5% ethanol solution before each behavioral test to eliminate possible odour clues left by the previous subject.

2.3.2. Gastric emptying of a solid meal in the mouse

Gastric emptying of a solid meal was measured as previously described (Wang et al., 2001) with slight modifications. Fasted mice (for 24 h with free access to water) were fed with access to water for a 1 h period, and then food was removed. Two hours later, mice were euthanized by cervical dislocation, the abdominal cavity was opened, and the stomach was removed and weighed. Then, the stomach was opened, its contents were washed out with distilled water, and the gastric wall was weighed. The amount (in grams) of food contained in the stomach was quantified as the difference between the weight of the stomach with and without its content. The amount of food ingested by the mice during the 1 h feeding period was determined by the difference between the total weight of the pellets before feeding and the weight of the pellets and spills at the end of the 1 h food exposure. The percentage of gastric emptying during the experimental period was calculated according to the following equation: gastric emptying (%) = [1 – (weight of food recovered from the stomach/weight of food intake)] × 100.

CRF and α-h-CRF were administered freehand intracerebroventricularly (i.c.v.) using the modified method of Haley and McCormick (1957) as described by Pedigo et al. (1975). For i.c.v. injections, mice were lightly anesthetized with ether and injections were made in the left lateral ventricle (coordinates 2 mm posterior and 2 mm lateral to the reference point bregma; depth 3 mm from the top of the skull) using a 10 μl Hamilton micro syringe. The solutions were delivered in a volume of 5 μl at the rate of 1 μl/s. The dose of 0.35 μg CRF was chosen on the basis of preliminary experiments showing its efficacy in inhibiting dose dependently (0.04–1.4 μg) gastric emptying. Etifoxine or its vehicle (10 ml/kg) was administered i.p. 30 min prior to the CRF injection while α-h-CRF was given concurrently with CRF. i.c.v. injections of CRF were performed at the time of the removal of food. As previously described in rats, lateral ventricular placement was verified by injecting 5 μl methylene blue solution (0.25 mg/ml) into the ventricle at the end of the experiment.

2.3.3. Data presentation and statistical analysis

All data are presented as means ± standard error of the mean (S.E.M.). The results from open field test and gastric emptying test were analysed with the procedure if the two conditions, normality of the data distribution and equality of the variances. Where appropriate, comparisons were made using the Student–Newman–Keuls test (SNK) or Dunn test, respectively, to evaluate the level of statistical significance between the different groups. Differences were considered statistically significant if P ≤ 0.05 (SigmaStat; v.3.1, SPSS Inc., USA).

2.4. CRF1 and CRF2 receptors binding assay

Rats were sacrificed by decapitation and whole brains (with cerebella) were extracted, dissected and placed in 30 volumes of ice-cold buffer (5 mM Tris–HCl, pH 7.4, 3 mM MgCl2, 1 mM EDTA, 0.25 M sucrose) then homogenized by several passes of a Teflon pestle homogenizer. The homogenate was centrifuged (1500 × g) at 4 °C for 15 min. The supernatant was discarded and the resulting pellet (P1) was suspended and homogenized in ice-cold 5 mM Tris–HCl buffer (pH 7.4) with 3 mM MgCl2, 1 mM EDTA and re-centrifuged as previously. The resulting supernatant was discarded and the pellet (P2) was treated like previously. The final pellet (P3) was re-suspended in 50 mM Tris–HCl buffer (pH 7.4) with 3 mM MgCl2 using a polytron. Protein concentration of each membrane preparation was determined by the method of Bradford (1976) with bovine serum albumin (0.1 mg/ml) as a standard.

Binding assays for [125I]h-CRF were carried out according to the slightly modified method of De Souza (1987). Aliquots of 100 μl of the membrane suspension (final protein concentration of 5–10 μg) were incubated in 50 μl of 50 mM Tris–HCl buffer (pH 7.4) containing 3 mM MgCl2, 0.01 mg/ml Leupeptine and 0.1% bovine serum albumin, with 50 μl of an [125I]h-CRF solution (final concentration of 0.1 nM) and 50 μl of solution with an appropriate concentration of CRF (chosen as reference ligand) or etifoxine. After 90 min incubation at 30 °C, the contents were rapidly filtered under vacuum through glass fiber filters (GF/C Whatman pre-soaked with 0.5% polyethyleneimine. The filters were then rapidly washed with 1 ml of ice-cold 10 mM Tris–HCl buffer (pH 7.4) containing 1 mM MgCl2, using a cell harvester (Brandel) and dried. Bound radioactivity (at the level of filters) was measured with a gamma counter (at 80% efficiency).

Specific radioligand binding was defined as the difference between total binding and non-specific binding (usually < 15% of the total binding) determined in the presence of unlabeled 1 μM CRF. For the competition curves between the radioligand and etifoxine (tested at five concentrations between 10− 8 and 10−5 M) or CRF (tested at six concentrations between 10−11 and 10−6 M), each separate experiment (at least n = 2) was performed in triplicate. Radioligand specific binding was expressed as the mean ± S.E.M. The concentration of compound that caused 50% inhibition of specific [125I]h-CRF binding (IC50 value) was determined by non-linear regression of the competition curves using Hill equation curve fitting. The inhibition constants (K) were calculated from the Cheng Prusoff equation (Piram program, GraphPad, San Diego, USA).

2.5. cAMP level measurements

Evaluation of the antagonist activity of etifoxine at the CRF1 and CRF2 receptors in human neuroblastoma SH-SYSY cells was determined according to the method described by Schoeffter et al. (1999) with minor modifications. The human neuroblastoma SH-SYSY cell line is described as endowed with native CRF1 and CRF2 receptors coupled to CAMP formation (Dieterich and De Souza, 1996; Hogg et al., 1996; Schoeffter et al., 1999). Intracellular accumulated cAMP was measured using the FlashPlate® radioimmunnoassay system (adenylyl cyclase activation assay system-kit SMP004, Perkin-Elmer, France). The assay was conducted in 96-well plates coated with a solid scinitillant to which anti-cAMP body was bound, and the total volume was 100 μl. Harvested cells were re-suspended at a density of 105 cells/ml in a stimulation buffer containing IBMX buffer (Dulbecco’s modified Eagle’s medium containing 2 mM 3-isobutyl-1-methylxanthine, 1 mg/ml bovine serum albumin, 35 mM Hepes–NaOH, pH 7.4). Then, 50 μl of cell suspension (5 × 105 cells) were distributed in each well and the volume was completed to 80 μl with phosphate buffer solution. Thereafter, the cells were incubated for 30 min at 22 °C with 30 nM CRF (10 μl) in the absence (control) or presence of the test compound or the reference antagonist α-h-CRF (10 μl). For basal control measurements, CRF was omitted from the reaction mixture. Thereafter, a detection buffer (100 μl) containing 0.18 μCi/ml [125I]CAMP was added and the mixture was further incubated for 2 h at 22 °C. Following incubation, the plates were counted for radioactivity in a scintillation counter (Topcount, Packard). It is assumed that the amount of cAMP produced by the cells is proportional to that of [125I]CAMP displaced from the anti-cAMP antibody.

The results, obtained from three independent experiments, each made in duplicate, were expressed as percent inhibition of the stimulated level of cAMP in presence of CRF. Etifoxine was tested at five concentrations ranging from 10−8 to 10−5 M. Results were given as mean ± S.E.M. The IC50 values (concentration causing a half-maximal inhibition of control values) were determined by non-linear regression analysis of the inhibition curves using Hill equation curve fitting (Sigmamak® v9.0; SPSS Inc., USA). In each experiment, the reference compound (α-h-CRF) was tested concurrently with etifoxine in order to assess the assay suitability. It was tested at eight concentrations (for IC50 value determination) and the data were compared with literature data.

3. Results

3.1. i.c.v. injection of CRF in the rodent

3.1.1. Open field test in the rat

The results with etifoxine in combination with CRF are illustrated in Fig. 1. The Kruskal–Wallis test and the ANOVA
revealed a significant general effect on the two parameters measured in the open field: grooming duration ($H(5) = 41.60, P < 0.001$) and number of squares crossed ($F(5,70) = 6.41, P < 0.001$), respectively. Post hoc analysis showed that CRF increased the duration of grooming ($P < 0.05$, Dunn test, Fig. 1A) and decreased the locomotion in the open field ($P = 0.008$, SNK, Fig. 1B) compared to the controls. Statistical analysis showed that $\alpha$-h-CRF significantly reduced CRF-induced grooming at the dose of 50 $\mu$g ($P < 0.05$, Dunn test) and at the same dose, significantly antagonized the locomotion–suppressing effects of CRF ($P = 0.029$, SNK). No significant differences in grooming duration ($P > 0.05$, Dunn test) and in locomotion ($P = 0.135$, SNK) were observed between pre-treated animals with $\alpha$-h-CRF alone at 50 $\mu$g and controls.

### 3.1.2. Gastric emptying of a solid meal in the mouse

Fig. 3 presents the effects of etifoxine in combination with CRF. ANOVA revealed a significant general effect on the

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**Fig. 1.** Effects of i.p. administration of various doses of etifoxine (EFX; mg/kg) on the modifications of the grooming duration (A) and of the number of crossed squares (B) induced by i.c.v. injection of 10 $\mu$g of CRF in the rat placed in the open field. Each column represents the mean ± S.E.M. The number of animals used appears at the bottom of each column. *$P < 0.05$, **$P < 0.01$ compared to the vehicle-treated controls (Veh). # $P < 0.05$ compared to CRF alone group.

**Fig. 2.** Effects of i.c.v. administration of various doses of $\alpha$-h-CRF (mg) on the modifications of the grooming duration (A) and of the number of crossed squares (B) induced by i.c.v. injection of 10 $\mu$g of CRF in the rat placed in the open field. Each column represents the mean ± S.E.M. of number of animals shown on bottom of columns. *$P < 0.05$ compared to the vehicle-treated controls (Veh). **$P < 0.05$ compared to CRF alone group.
gastric emptying ($F(5,84) = 9.31, P < 0.001$). Further comparisons showed that CRF significantly reduced gastric emptying ($P < 0.001$, SNK) compared with the controls. Etifoxine at the 50 mg/kg dose had no effect on its own ($P = 0.251$, SNK) but reduced the CRF-induced decrease in gastric emptying at the same dose ($P = 0.005$, SNK).

Fig. 4 shows the effects of α-h-CRF in combination with CRF. ANOVA revealed a significant general effect on gastric emptying ($F(5,54) = 12.48, P < 0.001$). Post hoc tests revealed a significant effect of 0.35 mg CRF ($P < 0.001$, SNK) in comparison with controls. Also similar to the etifoxine effects, α-h-CRF (at a dose of 7 μg) was devoid of effect on its own ($P = 0.379$, SNK) but attenuated dose-dependently the CRF-induced decrease in gastric emptying with a significant effect at doses of 3.5 and 7 μg ($P = 0.05$ and $P < 0.001$, respectively, SNK).

### 3.2. CRF$_1$ and CRF$_2$ receptors binding assay

Etifoxine displayed a negligible affinity for the CRF$_1$ and CRF$_2$ receptors since it inhibited the specific binding of [125I]-h-CRF less than 50% (8–34%) even at a high concentration of 10 μM (Table 1). Under the same experimental conditions, CRF exhibited concentration-dependent inhibition of [125I]-h-CRF binding with an IC$_{50}$ of 0.74 ± 0.01 nM ($K_i$ = 0.50 nM), and these findings are in accordance with those in the literature (De Souza, 1987).

### 3.3. cAMP level measurements

The basal level of cAMP was 52 ± 5 pmoles/ml ($n = 30$) and in presence of a 30 nM concentration of CRF, the amount of cAMP produced was increased two-fold to reach 119 ± 8 pmoles/ml ($n = 30$). Etifoxine inhibited the CRF-enhanced cAMP production with a weak efficacy: ~15% of control at 10 μM (IC$_{50}$ over 10 μM) (Table 1). By contrast, α-h-CRF reversed the CRF-induced stimulation of cAMP with an IC$_{50} = 272 ± 39$ nM. This value is in accordance with those of the literature (Schoeffter et al., 1999).

### 4. Discussion

In the present study, etifoxine attenuated the anxiogenic-like behavioral and autonomic responses to i.c.v. injection of CRF in the rodent. Thus, in the open field, rats receiving CRF responded with an increased sensitivity to the stressful aspects of a new situation. They remained in one of the corners of the arena, grooming or moving forwards and backwards. Increased grooming behavior has been related to fear or increased emotional-like response (Morley and Levine, 1982; Kalueff and Tuohimaa, 2004, 2005). The decrease by etifoxine of the CRF-induced grooming is interpreted as a manifestation of its anxiolytic effect and is consistent with the activity previously exhibited in classical anxiety animal models (Schlichter et al., 1999).
Etihozone also reversed the CRF-induced delay in gastric emptying of a solid meal. It is well known that exposure to stressful stimuli, mediated by the endogenous release of CRF, alters gastrointestinal motility resulting in decreased gastric secretion and emptying in experimental animals and humans (Coskun et al., 1997; Tacché et al., 2001). In the present study, α-h-CRF, a competitive antagonist of CRF₁ and CRF₂ receptors (Rivier et al., 1984) exhibited similar effects except that α-h-CRF reversed the CRF-induced hypo-locomotion in the open field.

The hypothesis that etifoxine might act as an antagonist at the CRF₁ and CRF₂ receptors was not supported by two sets of results: (i) the failure of etifoxine to reproduce the effects of α-h-CRF on CRF-induced hypo-locomotion and (ii) the results of binding and in vitro functional studies. Etihozone did not exhibit affinity for CRF₁ and CRF₂ binding sites (IC₅₀ > 10 μM), suggesting that this compound does not act directly at CRF receptors. The CRF₁ and CRF₂ receptors belong to the class II G-protein-coupled receptor super-family (Hilhouse et al., 2002). Functionally, in most tissues (e.g. brain), stimulation of these receptors by CRF and CRF-related peptides triggers activation of adeny cyclase and increases cAMP levels (Grammatopoulos et al., 1994; Grammatopoulos and Chrousos, 2002). To assess the potential antagonistic activity of etifoxine, the in vitro neuroblastoma cell system was suitable to investigate the functional consequences mediated through the CRF₁ and CRF₂ receptors. The fact that etifoxine inhibited the CRF-induced cAMP stimulation with a weak potency supports the notion that it does not behave as a functional antagonist of the CRF receptors.

There is precedent for anxiolytics with various mechanisms of action to counteract CRF-induced anxiety-like behavior in rodents without a real antagonistic activity on the CRF₁ and CRF₂ receptors (Lazosky and Britton, 1991; To et al., 1999). In vitro and in vivo pharmacological studies have suggested that drugs acting through the GABA/benzodiazepine/chloride channel receptor complex (such as benzodiazepines) may exert, at least in part, their anxiolytic and/or antidepressant effects via suppression of central CRF secretion (Calogero et al., 1988; Patchev et al., 1994; Serra et al., 1999; Cullinan, 2000). The well established etifoxine-induced enhancing of the GABAergic transmission by a positive allosteric effect via the chloride channel site (Verleye et al., 1999, 2002; Schlichter et al., 2000), may be linked to its inhibitory effect on the CRF system.

Recent data obtained with etifoxine suggest that facilitation of GABAergic inhibition may be also associated with an indirect mechanism implying activation of the peripheral benzodiazepine receptor with a subsequent increase in neurosteroid production (Schlichter et al., 2000), may be involved in CRF-clearance or degradation (Behan et al., 1996; Van Den Eede et al., 2005). A potential interaction between etifoxine and CRF-BP in the way of an up-regulation of this protein is a possibility worthy of further investigation.

In conclusion, the present study suggests that etifoxine administration-induced attenuation of the “anxiogenic” behavioral effects of CRF involves the GABAergic properties of this compound and does not support the hypothesis of a direct interaction between etifoxine and the CRF₁ and CRF₂ receptors.

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