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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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Keywords: Corticotropin-releasing factor; Anxiety; Etifoxine; Binding studies; cAMP assays; Rodent

1. Introduction

The corticotropin-releasing factor (CRF) also referred to as 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 adrenocorticotropin 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 (Pelleymounter 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

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etifoxine as the test compound. Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride, Stresam®), 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 γ-amino butyric acid$_A$ (GABA$_A$) 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 β_2 and β_3 subunits composing the GABA$_A$ 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, α-helical CRF$_{9–41}$ (α-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 × 43 cm × 19 cm) polypropylene 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 °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 α-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 α-h-CRF vehicle and CRF vehicle). For the binding assay, stock solutions (100 mM) 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 α-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 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 α-h-CRF or vehicle were intracerebroventricularly (i.c.v.) microinjected in 5 μl 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 α-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 μg 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 × 100 cm × 50 cm). The floor was divided into 25 identical sectors (20 cm × 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 − (wet 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/2 s.The dose of 0.35 μg CRF used 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 one-way analysis of variance (ANOVA) or the non-parametric Kruskal–Wallis procedure if the two conditions, normality of the data distribution and equality of variances, were not fulfilled. When appropriate, post hoc 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 $P \leq 0.05$ (SigmaStat; v.3.1, SPSS Inc., USA).

2.4. CRF_1 and CRF_2 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 $MgCl_2$, 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 $MgCl_2$, 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 $MgCl_2$ 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 [^{125}I]-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 $MgCl_2$, 0.01 mg/ml Leupeptine and 0.1% bovine serum albumin, with 50 μl of an [^{125}I]-hCRF 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 $MgCl_2$ 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^{-9} 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 [^{125}I]-h-CRF binding (IC_{50} value) was determined by non-linear regression of the competition curves using Hill equation curve fitting. The inhibition constants (K_i) were calculated from the Cheng Prusoff equation (Prism program, GraphPad, San Diego, USA).

2.5. cAMP level measurements

Evaluation of the antagonist activity of etifoxine at the CRF_1 and CRF_2 receptors in human neuroblastoma SH-SY5Y cells was determined according to the method described by Schoeffter et al. (1999) with minor modifications. The human neuroblastoma SH-SY5Y cell line is described as endowed with native CRF_1 and CRF_2 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® radioimmunoassay system (adenylyl cyclase activation assay system-kit SMP004, Perkin-Elmer, France). The assay was conducted in 96-well plates coated with a solid scintillant to which anti-cAMP body was bound, and the total volume was 100 μl.

Harvested cells were re-suspended at a density of 10^7 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 (5×10^5 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 [^{125}I] 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 [^{125}I] 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^{-9} to 10^{-5} M. Results were given as mean ± S.E.M. The IC_{50} 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 (Sigmaplot® 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 IC_{50} 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

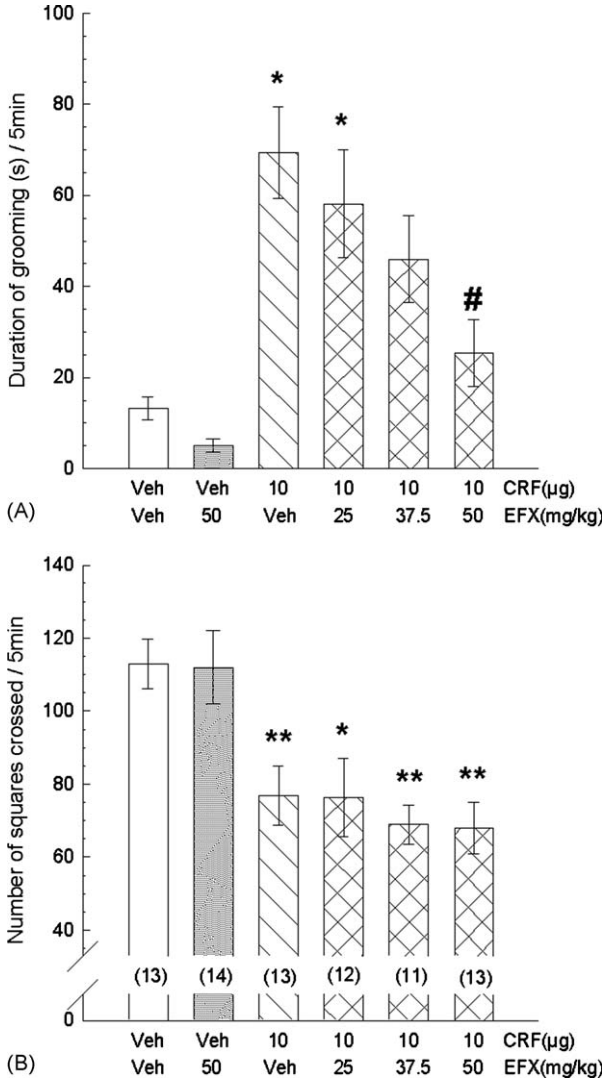

Fig. 1. Effects of i.p. administration of various doses of etifoxine (EFX; mg/kg) on the modifications of the grooming duration (A) and of the number of crossed squares (B) induced by i.c.v. injection of 10 μg of CRF in the rat placed in the open field. Each column represents the mean ± S.E.M. The number 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.

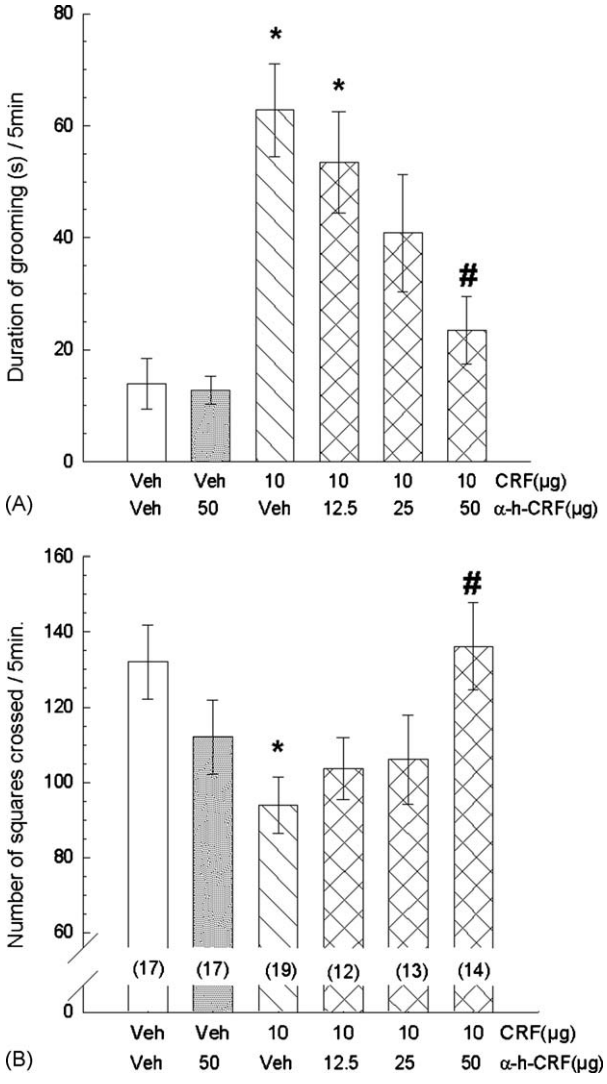

Fig. 2. Effects of i.c.v. administration of various doses of α-h-CRF (μg) on the modifications of the grooming duration (A) and of the number of crossed squares (B) induced by i.c.v. injection of 10 μg of CRF in the rat placed in the open field. Each column represents the mean ± S.E.M. of number of animals shown on bottom of columns. $^{*}P < 0.05$ compared to the vehicle-treated controls (Veh). $^{\#}P < 0.05$ compared to CRF alone group.

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. Etifoxine reversed the CRF-induced increase in grooming duration in an apparent dose-dependent fashion with a statistically significant effect reached at a dose of 50 mg/kg ($P < 0.05$, Dunn test). However, etifoxine up to the 50 mg/kg dose did not affect the CRF-induced decrease in locomotion ($P > 0.789$, SNK). Etifoxine, administered alone at the highest dose (50 mg/kg), did not modify either the grooming duration ($P > 0.05$, Dunn test) or the number of squares crossed ($P = 0.936$, SNK) compared to the controls.

Fig. 2 shows the results of the behavior of rats treated with α-h-CRF in combination with CRF. As previously, CRF produced an increase in the duration of grooming ($P < 0.05$, Dunn test) (Fig. 2A) and a decrease in the locomotion expressed by a reduction in the number of sectors crossed ($P = 0.032$, SNK) (Fig. 2B) compared to controls. Statistical analysis showed that α-h-CRF significantly reduced CRF-induced grooming at the dose of 50 μg ($P < 0.05$, 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 α-h-CRF alone at 50 μg 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

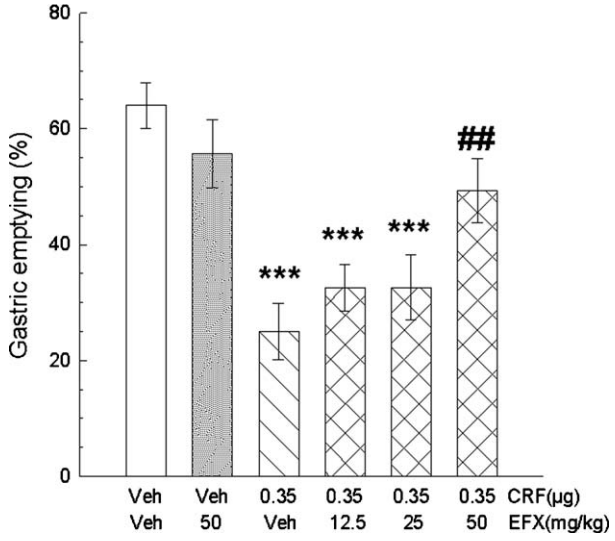

Fig. 3. Effects of i.p. administration of various doses of etifoxine (EFX-mg/kg) on CRF-induced inhibition (µg) of gastric emptying of a solid nutrient meal in mice. CRF was administered by i.c.v. route at a dose of 0.35 µg/animal. Each column represents the mean ± S.E.M. of 15 animals/group. $^{***}P < 0.001$ compared to the vehicle-treated controls (Veh). $^{\#\#}P < 0.01$ 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

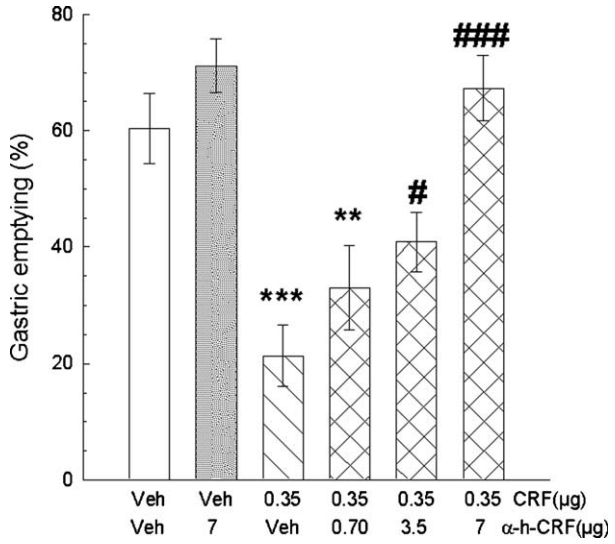

Fig. 4. Effects of i.c.v. administration of various doses of α-h-CRF on CRF-induced inhibition (µg) of gastric emptying of a solid nutrient meal in mice. CRF was administered by i.c.v. route at a dose of 0.35 µg/animal. Each column represents the mean ± S.E.M. of 10 animals/group. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the vehicle-treated controls (Veh). $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to CRF alone group.

Table 1
In vitro affinity of etifoxine (EFX) on the [^{125}I]-h-CRF binding to rat brain membranes and its effects on CRF-induced increase in cAMP accumulation in human neuroblastoma SH-SY5Y cells

Measures	IC_{50} (nM)	
	EFX	Reference compound
[^{125}I]-h-CRF binding inhibition	>10,000	0.74 ± 0.01 (r/hCRF)
cAMP production inhibition	>10,000	272 ± 39 (α-h-CRF)

Values represent the mean ± S.E.M. of at least two independent experiments, each done in duplicate in the binding and in the cAMP assays.

a significant effect of 0.35 µg 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 [^{125}I]-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 [^{125}I]-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 $IC_{50} = 272 \pm 39$ 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.,

2000; Verleye and Gillardin, 2004). Etifoxine 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; Taché et al., 2001). In the present study, α-h-CRF, a competitive antagonist of CRF_1 and CRF_2 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 CRF_1 and CRF_2 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. Etifoxine did not exhibit affinity for CRF_1 and CRF_2 binding sites ($IC_{50} > 10$ μM), suggesting that this compound does not act directly at CRF receptors. The CRF_1 and CRF_2 receptors belong to the class II G-protein-coupled receptor super-family (Hillhouse et al., 2002). Functionally, in most tissues (e.g. brain), stimulation of these receptors by CRF and CRF-related peptides triggers activation of adenyl 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 CRF_1 and CRF_2 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 CRF_1 and CRF_2 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; Verleye et al., 2005). These compounds, such as allopregnanolone (major metabolite of progesterone) are potent agonists of the $GABA_A$ receptor complex (Majewska et al., 1986) and it has been shown that the anxiolytic effects of allopregnanolone are mediated through interactions with hypothalamic CRF. For example, a study reported that allopregnanolone counteracted the anxiety state induced by exogenously administered CRF in rats (Patchev et al., 1994). Taken together, these observations support that potentiation of the activity of GABAergic inhibitory neurotransmission by etifoxine results in a decrease in CRF activity mediated by a direct effect on the chloride channel site coupled to the $GABA_A$ receptor and/or an indirect effect through neurosteroids. Investigations are currently planned to study the in vitro effects of etifoxine on CRF mRNA expression and on CRF biosynthesis. However, an other possible mechanism, unrelated to CRF system direct modulation, that could explain how etifoxine reduced the exogenous CRF-induced anxiogenic-like effects, can be suggested. Indeed, it has been shown that CRF stimulates brain serotonergic and noradrenergic systems (Kagamiishi et al., 2003; Murphy et al., 2003). Considering the numerous complex interactions between the different central specific neurotransmitters systems (see Millan, 2003), it can be proposed that etifoxine-enhanced inhibition of GABAergic pathways counteracts or exercises a braking effect on the CRF-induced hyperactivity of monoaminergic neurotransmission. In addition, the action of CRF in brain and in periphery can also be modulated by a binding protein (CRF-BP). CRF-BP, co-localized with CRF in the brain, is though to act as a negative regulator of the effects of CRF by modulating the availability of ''free'' CRF to interact with its receptors and 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 CRF_1 and CRF_2 receptors.

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