

Effects of etifoxine on ligand binding to GABA_A receptors in rodents

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Abstract

The GABA_A receptor/chloride ionophore is allosterically modulated by several classes of anxiolytic and anticonvulsant agents, including benzodiazepines, barbiturates and neurosteroids. Etifoxine, an anxiolytic and anticonvulsant compound competitively inhibited the binding of [³⁵S]γ-butyrbicyclopophosphoro-thionate (TBPS), a specific ligand of the GABA_A receptor chloride channel site. To investigate the etifoxine modulatory effects on the different binding sites of the GABA_A receptor complex, we have examined the effects of etifoxine on binding of the receptor agonist [³H]muscimol and the benzodiazepine modulator [³H]flunitrazepam in rat brain membrane preparations. The anticonvulsant properties of etifoxine combined with muscimol and flunitrazepam were performed in mice with picrotoxin-induced clonic seizures. Etifoxine modestly enhanced binding of [³H]muscimol and of [³H]flunitrazepam by increasing the number of binding sites without changing the binding affinity of [³H]flunitrazepam. In contrast, the compound decreased the affinity of muscimol for its binding site. In vivo, the combination of subactive doses of etifoxine with muscimol or flunitrazepam produced an anticonvulsant additive effect against the picrotoxin-induced clonic seizures in mice. These results suggest that the interaction of etifoxine on the GABA_A receptor complex would allosterically modify different binding sites due to conformational changes. Functionally, the resulting facilitation of GABA transmission underlies the pharmacological properties of etifoxine. © 2002 Elsevier Science Ireland Ltd and the Japan Neuroscience Society. All rights reserved.

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1. Introduction

Etifoxine, a non-benzodiazepine compound, has anxiolytic-like and anticonvulsant properties in rodents (Boissier et al., 1972; Kruse and Kuch, 1985) and is effective in the treatment of adjustment disorder with anxiety in humans (Servant et al., 1998). Etifoxine binds to a site distinct from that for benzodiazepines (BZDs) and competes, with a micromolar inhibition constant, with a ligand (TBPS) binding to the non-competitive channel blocker site of the GABA_A receptor complex (Verleye et al., 1999). Previous investigations of its interaction with the GABA_A receptor complex have led to a comparison of its effects with those of the neurosteroid, 3 α-hydroxy-5-α-pregnan-20-one or allopregnanolone, on GABA_A currents and gabaergic

synaptic transmission (Schlichter et al., 2000). These two compounds share common anxiolytic and anticonvulsant properties (Majewska, 1992; Gasior et al., 1999; Schlichter et al., 2000). Electrophysiological studies have also shown similarities between the facilitatory effect of etifoxine and that of allopregnanolone, on GABA_A currents and gabaergic synaptic transmission (Schlichter et al., 2000). On the other hand, in vitro binding studies suggest that etifoxine and allopregnanolone bind to distinct putative recognition sites at or near the chloride channel site (Verleye et al., 2001). Certain natural neurosteroids, such as allopregnanolone, can modulate the GABA_A receptor coupled chloride ionophore in a barbiturate-like manner (for review, see Gee, 1988) and possess properties associated with positive allosteric modulators of the GABA_A receptor. These include allosteric enhancement of agonist (for example, [³H]muscimol), and BZD (for example, [³H]flunitrazepam) binding to the receptor complex (Majewska et al., 1986; Harrison et al., 1987). For these effects, the potency of neurosteroids tends to be in the

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nanomolar range while that of volatile anesthetics and barbiturates is in the micro or millimolar range (Harris et al., 1994). Generally, these compounds act through a change in the apparent dissociation constant (K_d) of [^3H]muscimol and [^3H]flunitrazepam rather than in the apparent maximal binding capacity (B_{max}) of radioligand binding.

In order to characterize etifoxine among the variety of compounds exerting similar effects at the GABA_A receptor, the present study had two objectives: (i) an investigation of the in vitro modulatory effect of etifoxine on the binding characteristics of [^3H]muscimol and [^3H]flunitrazepam in rat cerebral cortex; (ii) an evaluation of the in vivo functional effects of the combination of etifoxine with muscimol or flunitrazepam in picrotoxin-induced convulsions in mice. The convulsant picrotoxin is a non-competitive inhibitor of GABA neurotransmission which binds to an independent site closely associated with the chloride ionophore (Simmonds, 1980; Gee, 1988).

2. Methods

2.1. Radioligand binding assay

For each binding assay the procedure, as outlined in literature, was followed. The experimental conditions were optimal in order to measure the specific binding of the radioligand to its receptor site at the equilibrium.

2.1.1. [^3H]muscimol binding assay

Preparation of rat brain cortical membranes and the [^3H]muscimol assay were performed at 4 °C as previously described (Snodgrass, 1978). Briefly, cortices were rapidly removed following decapitation of male Sprague Dawley rats (150–200 g), homogenized in 20 volumes 0.32 M sucrose and the P1 pellet collected after a 1000 × *g* centrifugation was discarded. The P2 pellet was collected after 20 min centrifugation at 20 000 × *g*. This pellet was resuspended in water, the suspension frozen overnight and after thawing, the membrane pellet was collected by centrifugation for 30 min at 45 000 × *g*. This pellet was washed with 25 mM Hepes-tris buffer, pH 7.0 and again centrifuged at 45 000 × *g*. Finally, membranes were added to 40 mM HEPES-tris buffer, pH 6.8. The protein concentration was determined according to the method of Bradford (1976). Incubations were done at 4 °C for 10 min with a final volume of 1.05 ml, after adding membranes (100–200 µg protein), drug to be studied, buffer, and finally [^3H]muscimol, at a final-concentration of 5 nM, for the competition curve and at nine different concentrations (1–30 nM) for the saturation curves. Following incubation, the cortical membranes were rapidly filtered under vacuum through glass fiber filters (GF/C Whatman). The filters were then

rapidly (15 s) washed three times with 1.5 ml of 25 mM Hepes-tris buffer, pH 7.0 using a cell harvester (Brandel). Bound radioactivity was measured with a scintillation counter (LS 6000, Beckman) using a liquid scintillation cocktail (formula 989, Packard) after standing overnight at room temperature.

Specific radioligand binding was defined as the difference between total binding and non-specific binding determined in the presence of unlabeled 10 µM muscimol (usually < 20% of the total binding).

2.1.2. [^3H]flunitrazepam binding assay

Preparation of rat cortical membranes and the [^3H]flunitrazepam assay were performed as previously described (Speth et al., 1979). Male Sprague Dawley rats (150–200 g) were sacrificed by decapitation and cortices were rapidly removed. Cerebral cortex was homogenized in 19 vol. (w/v) of ice cold 50 mM phosphate (NaKPO₄: 81 mM Na⁺, 9.5 mM K⁺, 50 mM PO₄) buffer with a Polytron. The homogenates were centrifuged at 48 000 × *g* for 10 min at 4 °C and the supernatants were discarded. Tissue pellets were resuspended in 19 volumes of NaKPO₄ buffer with a Polytron homogenizer. Protein content of the cortex homogenates was determined by the method of Bradford (1976). Aliquots (50 µl) of the brain membrane suspension were incubated with 0.4 nM [^3H]flunitrazepam in a final volume of 2.0 ml NaKPO₄ buffer for 60 min at 4 °C. The Scatchard plots were based on nine different concentrations of ligand (0.2–16 nM).

At the completion of the incubation, the cortical membranes were rapidly filtered under vacuum through glass fiber filters (GF/B or GF/C Whatman) and washed three times with 5 ml of ice-cold NaKPO₄ buffer using a cell harvester (Brandel).

The radioactivity retained by the filters was measured with a scintillation counter (LS6000, Beckman) using a liquid scintillation cocktail (formula 989-Packard). Specific radioligand binding was defined as the difference between total binding and non-specific binding determined in the presence of unlabeled 3 µM diazepam (ranged from 10 to 15% of total binding).

2.1.3. Data analysis

For the competition curve between the radioligand and etifoxine, each independent experiment (at least *n* = 2) was performed in triplicate. Radioligand specific binding data are expressed as the mean ± SEM. For the Scatchard analysis, three independent saturation binding curves were obtained by incubating aliquots of membrane homogenates with several increasing concentrations of the radioligand in the absence of any compound (control total binding), in the presence of a high concentration of unlabeled ligand (non-specific binding), and in the presence of a single concentration of etifoxine chosen to increase sharply the binding of the

specific ligand. The radioligand equilibrium dissociation constant (K_d) and the binding site density (B_{max}) were calculated by computer-assisted non-linear regression of bound versus free radioligand-concentration (Sigma plot, v.4.1, Jandel Scientific, GmbH). Values are given as the mean \pm SEM of three experiments, with each experiment performed in duplicate.

Mean values were compared using Student's paired two-tailed *t*-test and *P*-values < 0.05 were considered statistically significant.

2.2. *In vivo* pharmacology

2.2.1. Animals

Male CD1 (ICR) mice weighing between 25 and 30 g were used. Upon arrival, they were housed in standard polypropylene cages (25/cage) in a room at constant temperature (22 ± 2 °C), with a 12 h/12 h (lights on from 7:00 h to 19:00 h) light/dark cycle and with a controlled humidity ($50 \pm 20\%$). Food (UAR-A04) and water (tap water) were freely available. Animals were acclimated for a minimum of 4 days prior to experimentation. The treatment of the animals and their care were conducted in accordance with the European Community Council Directive 86/609/EEC.

2.2.2. Measurement of seizure threshold

Picrotoxin (0.5%; w/v) was infused into the tail vein of lightly restrained mice at a constant rate (0.3 ml/min) with an infusion pump (model PHD 2000; Harvard apparatus). The onset of clonus affecting the whole body was used as the endpoint. The time to convulsion was measured and from this value the dose or threshold in mg/kg required to produce the convulsion was calculated. The total infused volume never exceeded 0.3 ml to avoid discomfort to the animal.

2.2.3. Pharmacologic procedure

Etifoxine was injected intraperitoneally (i.p.) 35 min before the infusion of picrotoxin whereas muscimol and flunitrazepam were injected i.p. 30 min before the convulsant drug. Controls received the same volume of vehicles. In the case of combination, the injected doses of etifoxine, muscimol and flunitrazepam were subactive.

2.2.4. Data analysis

Threshold values were expressed as mean \pm SEM. When the compounds were administered alone, statistical analysis used the one-way analysis of variance (ANOVA) or the Kruskal Wallis test followed post-hoc by Dunnett's test or Dunn's test with the vehicle group as reference. In the case of the combination of the two compounds at subactive doses, the one-way ANOVA or the Kruskal Wallis procedure followed post hoc by the Student Neuman–Keuls multiple comparison test or

Dunn's test was performed to locate the differences between the experimental groups. The criterion for statistical significance was $P < 0.05$ (Sigmastat, v.2.0, Jandel Scientific, GmbH).

2.2.5. Drugs used

For binding assays, stock solutions (100 mM) of etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride; batch 46, Biocodex Laboratoires, France) were made up in dimethyl sulfoxide (DMSO) and diluted to the desired concentration with distilled water (final concentration of DMSO $< 0.1\%$). Radiolabeled ligands and other substances were obtained from New England Nuclear (France) and Sigma (France), respectively.

For *in vivo* studies, etifoxine and flunitrazepam (Rohypnol®) were suspended in saline solution (0.9% NaCl) with 1% Tween 80 (v/v). Muscimol (Sigma-France) was dissolved in saline solution. Picrotoxin (Sigma-France) was dissolved in DMSO (final concentration of DMSO = 4% V/V) and then diluted in saline. At this concentration infused DMSO was devoid of any effect in the animal. All drugs were administered i.p. in a volume of 0.1 ml/10 g.

3. Results

3.1. Binding studies

3.1.1. Effects of etifoxine on [3 H]muscimol binding

Etifoxine (EFX), at concentrations ranging from 10 to 300 μ M (higher concentrations limited its solubility), produced a dose-dependent increase in the [3 H]muscimol binding at equilibrium, to $155 \pm 2\%$ of its control value at 300 μ M EFX (Fig. 1A).

From Scatchard plots (Fig. 1B), EFX (300 μ M) significantly increased the B_{max} of the ligand compared with control (733 ± 167 vs. 355 ± 81 fmol/mg protein; $P < 0.05$) and the K_d of the ligand compared with control (23.0 ± 3.5 vs. 10.5 ± 0.8 nM; $P < 0.05$).

3.1.2. Effects of etifoxine on [3 H]flunitrazepam binding

At concentrations ranging from 0.1 to 300 μ M, EFX modestly enhanced [3 H]flunitrazepam binding with a maximal enhancement ($125 \pm 8\%$ of control) at 100 μ M. A concentration-dependent effect of EFX was noted (Fig. 2A).

In saturation experiments (Fig. 2B), EFX 300 μ M significantly increased the B_{max} of [3 H]flunitrazepam (1280 ± 92 vs. 969 ± 161 fmol/mg protein for control, $P < 0.05$) with no change in the K_d (2.9 ± 1.0 vs. 2.2 ± 0.3 nM for control, $P > 0.05$).

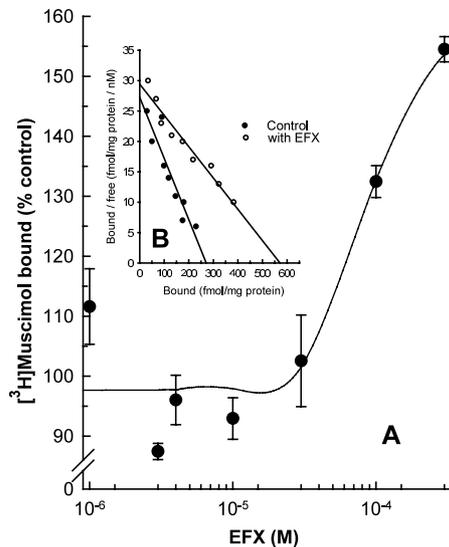


Fig. 1. A. Concentration-dependent enhancement of specific [^3H]muscimol binding from rat cerebral cortex membranes by etifoxine (EFX). Each point is the mean \pm SEM of least two independent experiments, each carried out in triplicate. B. Scatchard plots of [^3H]muscimol binding in rat cerebral cortex membranes in the absence and presence of 300 μM etifoxine (EFX). The data are from one experiment performed in duplicate and are representative of three such experiments with similar results.

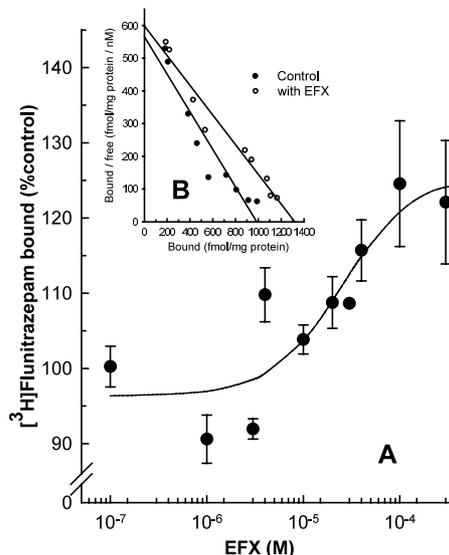


Fig. 2. A. Concentration-dependent enhancement of specific [^3H]flunitrazepam binding from rat cerebral cortex membranes by etifoxine (EFX). Each point is the mean \pm SEM of least two independent experiments, each carried out in triplicate. B. Scatchard analysis of [^3H]flunitrazepam binding in the absence and presence of 300 μM etifoxine (EFX). The data are from one experiment performed in duplicate and are representative of three such experiments with similar results.

3.2. In vivo pharmacology

3.2.1. Effects of drugs alone on the seizure threshold

Etifoxine, muscimol and flunitrazepam increased the seizure threshold dose-dependently at minimal signifi-

cant effective doses of 75, 1 and 0.3 mg/kg, respectively (Fig. 3A, B, C).

3.2.2. Effects of combined drugs on the seizure threshold

Combined subactive doses of etifoxine (50 mg/kg) and muscimol (0.5 mg/kg) or flunitrazepam (0.15 mg/kg) significantly enhanced the doses of picrotoxin producing clonic seizures compared to the values measured for controls and for animals treated with the compounds given alone (Fig. 4A, B).

4. Discussion

Potential of GABA action at GABA_A receptors is considered as the most important molecular event underlying the anxiolytic and anticonvulsant effects of BZDs, barbiturates, and neurosteroids (Breier and Paul, 1990; Mehta and Ticku, 1999). Etifoxine is a clinically useful anxiolytic but its mechanism of action is not well understood. Recent studies have shown that it exerts its effect by interacting with the chloride channel of GABA_A receptors and probably by facilitating GABAergic inhibition (Verleye et al., 1999; Schlichter et al., 2000). From binding studies using [^{35}S]TBPS, which binds to a site on or near the chloride ionophore (Squires et al., 1983), it has been shown that etifoxine competitively inhibits [^{35}S]TBPS binding with micromolar potency in rat brain. In vivo studies demonstrated an anticonvulsant effect of etifoxine against the clonic convulsions induced by TBPS in mice (Verleye et al., 1999). This effect was not blocked by flumazenil, an antagonist of BZDs sites at GABA_A receptors, suggest-

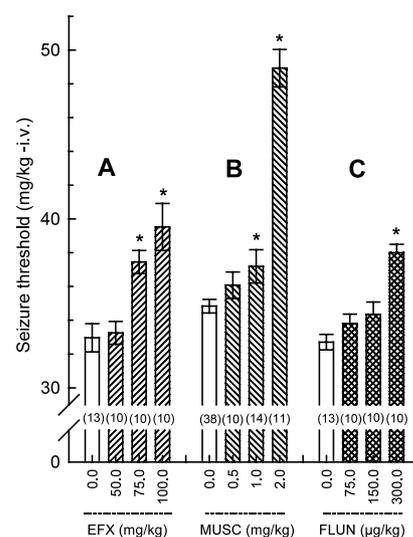


Fig. 3. The effect of various doses of etifoxine (EFX) (A) muscimol (MUSC) (B) and flunitrazepam (FLUN) (C) on the seizure threshold measured by infused picrotoxin in mice. Each column represents the mean \pm SEM. The number in brackets is the number of animals used. * $P < 0.05$ compared to controls.

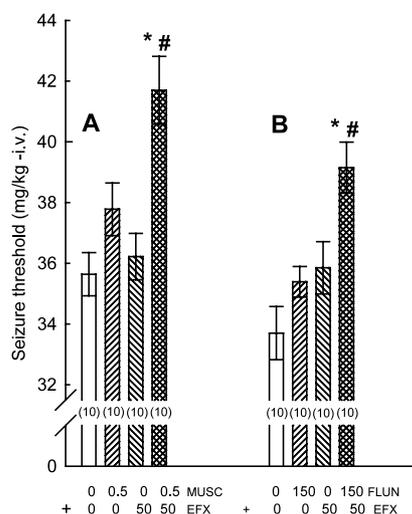


Fig. 4. Effects of etifoxine (EFX, mg/kg) combined with muscimol (MUSC, mg/kg) (A) and flunitrazepam (FLUN, μ g/kg) (B) at subactive doses on the seizure threshold measured by infused picrotoxin in mice. Each column represents the mean \pm SEM. The number of animals used is in brackets. * $P < 0.05$ compared with controls (doses 0). # $P < 0.05$ significantly different from MUSC or FLUN alone.

ing that etifoxine may bind to a site distinct from that for BZDs. The present in vitro results reveal a complex interaction of etifoxine with the GABA_A receptor complex. Etifoxine did not displace either [³H]muscimol or [³H]flunitrazepam binding from rat cortical membrane preparations. Conversely, it slightly enhanced the binding of these radioligands on their respective sites. Scatchard analysis of the radioligands binding data showed that etifoxine, at 300 μ M, increased the number of binding sites (B_{max}) of both [³H]muscimol and [³H]flunitrazepam without altering the apparent affinity of [³H]flunitrazepam but decreasing the apparent affinity (or increasing K_d) of [³H]muscimol. The GABA_A receptor is an oligomeric protein complex, consisting of several sub-units with independent but interacting binding sites including the GABA recognition site. These binding sites bear an allosteric relationship to each other (Ticku, 1991; Mehta and Ticku, 1999). We hypothesize that when etifoxine binds to one or more subunits of the receptor, it perturbs the structure of the whole protein complex, causing changes in the other binding sites, e.g. recruitment of novel binding sites with consequently an altered affinity for other binding sites. In the in vivo functional studies, etifoxine, muscimol and flunitrazepam showed anticonvulsant efficacy against picrotoxin-induced clonic seizures by increasing the amount of the convulsant required to produce convulsions in mice. The combination of an etifoxine sub-active dose with a sub-active dose of muscimol or flunitrazepam produced an additive effect. The in vitro effects of etifoxine on the radioligands binding were observed at relatively high concentrations (> 100 μ M).

Although etifoxine plasma unbound or brain concentrations were not measured, it appears that there is consistency between the in vitro and in vivo findings of this study. Taken together, these data suggest that etifoxine enhances GABAergic function by a positive allosteric modulation of the GABA_A receptor. Many drugs, such as barbiturates, general anesthetics and neurosteroids, with anxiolytic, anticonvulsant and hypnotic properties exert similar effects at the GABA_A receptor complex. These compounds inhibit the binding of TBPS to the chloride ionophore and enhance the binding of GABA agonists, such as muscimol and flunitrazepam (Majewska et al., 1986; Tobin, 1991; Harris et al., 1994; Wieland et al., 1997). The potency of neurosteroids is in the nanomolar range while that of volatile anesthetics and barbiturates is in the micro or millimolar range. Generally, these compounds act through a change in K_d of [³H]muscimol and [³H]flunitrazepam rather than in B_{max} of radioligand binding. For example, one of the major effects of propofol, a general anesthetic, on [³H]muscimol binding is to increase the affinity of the lower affinity sites for muscimol without changing the total binding capacity (Davies et al., 1998). However, compounds such as inhalational anesthetics (Harris et al., 1994) or others such as etomidate or pentobarbital (Thyagarajan et al., 1983) enhance GABA_A agonists and BZDs binding to rodent cerebral cortex membranes by an increase in B_{max} of radioligand binding without affecting K_d . A possible apparent unmasking of low affinity sites of ligands to the GABA_A receptor complex has been proposed (Harris et al., 1994). The subunit composition of the GABA_A receptor has important functional implications for the modulatory effects of the above compounds (reviewed in Sieghart, 1995; Maitra and Reynolds, 1999; Rudolph et al., 2001). Future studies with etifoxine will focus on identification and characterization of the subunits of the GABA_A receptor associated with its effects.

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