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Neuropharmacology 45 (2003) 293–303
NEURO PHARMACOLOGY
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# The modulatory effects of the anxiolytic etifoxine on $GABA_A$ receptors are mediated by the β subunit

Alain Hamon [a,*], Alain Morel [b], Bernard Hue [a], Marc Verleye [c], Jean-Marie Gillardin [c]

[a] *Laboratoire de Neurophysiologie, UPRES EA 2647 (RCIM), Université d'Angers, UFR Sciences, 2 Boulevard Lavoisier, F-49045 Angers cedex 01, France*
[b] *Laboratoire d'Oncopharmacologie, UPRES EA 3140, Centre Paul Papin, 2 rue Moll, F-49033 Angers cedex 01, France*
[c] *Laboratoires Biocodex, Centre de recherche, 60200 Compiègne, France*

Received 19 March 2002; received in revised form 21 March 2003; accepted 24 March 2003

**Abstract**

The anxiolytic compound etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4*H*-3,1-benzoxazine hydrochloride) potentiates $GABA_A$ receptor function in cultured neurons (Neuropharmacology 39 (2000) 1523). However, the molecular mechanisms underlying these effects are not known. In this study, we have determined the influence of $GABA_A$ receptor subunit composition on the effects of etifoxine, using recombinant murine $GABA_A$ receptors expressed in *Xenopus* oocytes. Basal chloride currents mediated by homomeric β receptors were reduced by micromolar concentrations of etifoxine, showing that β subunits possess a binding site for this modulator. In oocytes expressing $\alpha_1\beta_x$ $GABA_A$ receptors ($x$ = 1, 2 or 3), etifoxine evoked a chloride current in the absence of GABA and enhanced GABA (EC10)-activated currents, in a dose-dependent manner. Potentiating effects were also observed with $\alpha_2\beta_x$, $\beta_x\gamma_{2s}$ or $\alpha_1\beta_x\gamma_{2s}$ combinations. The extent of potentiation was clearly β-subunit-dependent, being more pronounced at receptors containing a $\beta_2$ or a $\beta_3$ subunit than at receptors incorporating a $\beta_1$ subunit. The mutation of Asn 289 in the channel domain of $\beta_2$ to a serine (the homologous residue in $\beta_1$) did not significantly depress the effects of etifoxine at $\alpha_1\beta_2$ receptors. This specific pattern of inhibition/potentiation was compared with that of other known modulators of $GABA_A$ receptor function like benzodiazepines, neurosteroids, barbiturates or loreclezole.


*Keywords:* Etifoxine; $GABA_A$ receptor; β Subunit; Allosteric modulator; Anxiolytic; *Xenopus* oocyte

## 1. Introduction

In the mammalian brain, the inhibitory activity of type A gamma-aminobutyric acid receptors ($GABA_A$Rs) can be potently enhanced by allosteric modulators such as benzodiazepines, neurosteroids or barbiturates. This property underlies the pharmacological treatment of diverse neuropsychiatric disorders like anxiety, insomnia or muscular spasms. Unfortunately, classical benzodiazepines display major side effects including anterograde amnesia, impairment of locomotor activity, ethanol-potentiation, tolerance and rebound effects on cessation of treatment (Woods and Winger, 1995). In contrast, neurosteroids exhibit less detrimental side effects but are not yet exploited on a large scale for their therapeutic potential (Gasior et al., 1999). Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4*H*-3,1-benzoxazine hydrochloride, Stresam®), a molecule structurally unrelated to benzodiazepines and neurosteroids, has also revealed anxiolytic properties in rodents (Boissier et al., 1972; Schlichter et al., 2000) and humans (Servant et al., 1998), without sedative, myorelaxant and mnesic side effects at anxiolytic concentrations (Micallef et al., 2001).

Recent binding and electrophysiological experiments have demonstrated that etifoxine binds to $GABA_A$Rs via an allosteric site which differs from that of benzodiazepines and neurosteroids (Verleye et al., 1999; Schlichter et al., 2000; Verleye et al., 2001, 2002). In addition, etifoxine increases GABAergic neurotransmission by an indirect mechanism involving the activation of periph-

* Corresponding author. Tel.: +33-241-735-442; fax: +33-241-735-215.
*E-mail address:* alain.hamon@univ-angers.fr (A. Hamon).

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doi:10.1016/S0028-3908(03)00187-4

eral (mitochondrial)-type benzodiazepine receptors and, very likely, an enhancement of neurosteroid synthesis (Schlichter et al., 2000).

Mammalian $GABA_A$Rs are hetero-pentamers assembled from distinct subunit families with multiple subtypes ($\alpha_{1-6}$, $\beta_{1-3}$, $\gamma_{1-3}$, $\delta$, $\epsilon$, $\pi$ and $\theta$). The most abundant native receptors, however, are composed of only $\alpha$, $\beta$ and $\gamma$ subunits with presumably a $2\alpha$:$2\beta$:$1\gamma$ stoichiometry (Sieghart et al., 1999; Mehta and Ticku, 1999; Knight et al., 2000). The subunit composition of $GABA_A$Rs has important functional implications for the effects of positive modulators like benzodiazepines and neurosteroids. Both $\alpha$ and $\gamma$ subunits are required for the high-affinity binding of benzodiazepines, thus suggesting that the binding site is located at the $\alpha$–$\gamma$ interface (Sigel and Buhr, 1997). By contrast, the modulation of GABA responses by neurosteroids does not require the presence of $\alpha$ or $\gamma$ subunits but depends on the presence of a $\beta$ subunit (Mehta and Ticku, 1999). The location of the binding site of etifoxine and its mechanism of action are unknown at present. In order to address these questions, as a first step, we used a recombinant strategy to determine what types of subunits underlie its modulatory effects. For this purpose, we analysed the ability of etifoxine to modulate GABA-evoked chloride currents in *Xenopus* oocytes expressing one ($\beta_x$), two ($\alpha_1\beta_x$, $\beta_x\gamma_{2s}$) or three ($\alpha_1\beta_x\gamma_{2s}$) murine $GABA_A$R subunits. The $\alpha_1$ and $\gamma_{2s}$ subunits were selected for co-expression with $\beta$ subunits because they are the most abundant representatives of their subunit family in many regions of the rodent brain and, therefore, are likely to co-assemble with a $\beta$ subunit to produce native $GABA_A$R subtypes (Pirker et al., 2000). In complementary experiments, we also examined the effects of replacement of $\alpha_1$ by $\alpha_2$ and the effects of mutating $\beta_2$ N-289, a residue that is known to control the interaction of several other positive modulators with $GABA_A$ receptors (Wafford et al., 1994; Belelli et al., 1997; Halliwell et al., 1999).

## 2. Materials and methods

### 2.1. Preparation of cloned cDNAs

cDNAs encoding the murine $\alpha_1$, $\beta_{1-3}$ and $\gamma_{2s}$ $GABA_A$R subunits, subcloned into the pGW1 (=pRK5) vector downstream of the cytomegalovirus promoter, were provided by Pr. S.J. Moss (University College, London, UK). The rat $\alpha_2$ and $\beta_{2(N289S)}$ $GABA_A$R cDNAs, which encode the same protein as their murine homolog, were provided by Pr. H. Lüddens (University of Mainz, Mainz, Germany) and Pr. N.L. Harrison (Cornell University, New York, USA), respectively. The identity of the wild-type and mutated $\beta_2$ subunits was verified by sequencing. Plasmids were introduced into the *E. coli* host strain XL1 blue by electroporation and propagated according to standard protocols. After amplification, plasmid DNAs were extracted and purified using a Qiagen kit and resuspended in an injection buffer of the following composition (in mM): NaCl 30, EDTA 0.5, HEPES 15, pH 8. The desired combinations of plasmids were mixed in equal ratios and DNA concentration was adjusted to avoid excessive expression levels ($\alpha\beta\gamma$ mixtures) or, on the contrary, excessively small membrane currents ($\beta\gamma$ mixtures).

### 2.2. Preparation of oocytes

Female *Xenopus laevis* frogs were anaesthetized in ice-cold water containing 0.15% tricaine (3-aminobenzoic acid ethyl ester, Sigma). Ovarian lobes were cut off through a small abdominal incision and thoroughly washed in standard oocyte saline (SOS) composed of (in mM): NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5, pH 7.5. Stage V–VI oocytes were partially defolliculated by enzymatic treatment with 2 mg/ml collagenase (type IA, Sigma) in calcium-free SOS for 10–15 min. This treatment was followed by manual dissection using fine forceps to achieve complete removal of the follicular envelopes. Ten to twenty nanoliters of the desired mixture of plasmid DNAs were injected into the animal pole of oocytes by the "blind method" of Colman (1984), using a digital microdispenser (Drummond Nanoject). Following injection, oocytes were stored at 17–18°C in a sterile medium consisting of SOS supplemented with gentamycin (50 μg/ml), penicillin (100 UI/ml), streptomycin (100 μg/ml), sodium pyruvate (2.5 mM) and horse serum (1–5%). The incubation medium was replaced every 2 days.

### 2.3. Electrophysiology

One to four days after injection, oocytes were tested for $GABA_A$R expression, using a two-electrode voltage clamp amplifier (Geneclamp 500, Axon Instruments), at a holding potential of −60 mV. They were held by four fine pins in a 100 μl-chamber and continuously superfused with SOS. The voltage sensing electrodes and the current passing electrodes were filled with 2 M KCl and had resistances of 1–5 MΩ when measured in SOS. Currents were displayed on a dual channel chart recorder (Gould BS-232).

In all experiments, agonists and antagonists were applied via the superfusate at a flow rate close to 4 ml/min. As etifoxine (Biocodex, France) is poorly soluble in SOS, it was first dissolved in dimethylsulfoxide (DMSO) just before use. The stock solution (100 mM) was then diluted in SOS to the required final concentration (0.1–20 μM). Electrophysiological recordings were not affected by DMSO at its final concentration (<0.1% v/v). In all experiments investigating the concentration dependence of the potentiation of GABA-

evoked currents, etifoxine was pre-applied for 2 min before co-application with a EC10 concentration of GABA, until the current response was observed to peak. Etifoxine was not tested at concentrations higher than 20 μM because its solubility is only partial from 30 μM. Applications of GABA were separated by intervals of 10–15 min to permit resolution of receptor desensitisation. Oocytes used to collect the data were mostly limited to those that had 10 nA–1 μA in response to EC10 GABA. Within that range, the quantitative effects of etifoxine did not appear to vary significantly with the level of expression. Picrotoxinin and GABA were obtained from Sigma (L'Isle d'Abeau Chesnes, France).

### 2.4. Data analysis

For each subunit combination, the EC10 concentration of GABA was determined by fitting the dose–response relationships to the logistic equation $I/I_{max} = C^n/(C^n + EC50^n)$, where $I$ represents the peak current recorded at concentration $C$, with $I_{max}$ being the maximal response and $n$ the Hill coefficient. The maximal current was defined using 3 mM GABA and the best fit to the data was obtained using a non-linear least-square fitting program (Prism 2.0, Graphpad Software).

The relative efficacy of 20 μM etifoxine in potentiating GABA responses was analysed for different subunit combinations. Pooled data are presented as mean ± SEM of 4–11 independent experiments. Statistical significance was determined at the $P < 0.05$ level using ANOVA or unpaired $t$-tests (Prism 2.0, Graphpad Software).

## 3. Results

### 3.1. Homomeric receptors

In accordance with earlier reports (Connolly et al., 1996a and b; Krishek et al., 1996; Wooltorton et al., 1997; Cestari et al., 1996, 2000; Sugimoto et al., 2000), none of the murine β subunits formed GABA-sensitive receptors when expressed alone in *Xenopus* oocytes. However, most β cDNA-injected cells displayed a persistent inward chloride current that could be blocked by 100 μM picrotoxinin (PTX), indicating that β subunits can oligomerize to form spontaneously opening homomeric receptor-channels (illustrated in Fig. 1 for $\beta_2$). While $\beta_1$ and $\beta_3$ subunits expressed reliably, the expression of $\beta_2$ appeared more erratic, as already mentioned by Cestari et al. (1996).

Homomeric β receptor channels offered the opportunity to test the effects of etifoxine in the absence of α and γ subunits. Whatever the β subunit used for expression, application of etifoxine produced a dose-dependent outward current characterized by a slow attainment of steady-state and incomplete recovery, even after a 20 min wash (Fig. 1A). Brief hyperpolarizing voltage pulses (10 mV, 10 s) superimposed on the holding potential (−60 mV) in the absence and in the presence of 20 μM etifoxine indicated that the outward current was due to a decrease in membrane conductance (Fig. 1B). Therefore, the real effect of etifoxine was not to activate an outward current but to inhibit an inward background current. In control experiments, etifoxine had no effect on uninjected oocytes or injected oocytes bathed with 100 μM picrotoxinin (right part of Fig. 1B), thus showing that its molecular target was the homomeric β GABA receptor. Such inhibitory effects of etifoxine were unexpected, since only potentiating effects have been reported on native $GABA_A$ receptors.

Outward currents were measured at steady-state (following incubation with etifoxine for 4 min) and concentration–response curves were constructed by plotting the percentage of inhibition (i.e. amplitude of the etifoxine-induced outward current normalized to the amplitude of the PTX-sensitive background current) versus concentration of etifoxine. Fig. 1C shows that the effects of etifoxine were slightly subunit-dependent, the extent of inhibition being greater (by ~1.7-fold in the presence of 20 μM etifoxine) for $\beta_2$ receptors than for the two other receptors.

### 3.2. Heteromeric $\alpha_1\beta_x$ receptors

The above results suggest that all three β subunits possess a binding site for etifoxine. As occupancy of this site led to inhibition of receptor function, it can be hypothesized that the potentiating effects reported for neuronal preparations reflect the influence of α or γ subunits. As single α or γ subunits or αγ combinations failed to produce functional receptors, the only possibility to address this question was to co-express a β subunit with an α or a γ subunit. The $\alpha_1$ and $\gamma_2$ subunits were selected for co-expression with $\beta_1$, $\beta_2$ or $\beta_3$ as they are the most abundant non-β subunits in many regions of the brain (Pirker et al., 2000).

All $\alpha_1\beta_1$ subunit combinations expressed efficiently and formed GABA-sensitive channels. In most experiments, a permanent inward current sensitive to PTX was also generated, showing that at least some of the expressed channels were spontaneously open. Application of etifoxine produced a dose-dependent inward current characterized by slow kinetics (Fig. 2A) and return to basal level upon washing (Fig. 2B). This current might correspond either to a potentiation of the basal current or to a direct agonist effect of etifoxine. Since inward currents were also produced in oocytes lacking PTX-sensitive spontaneous currents (Fig. 2B), it appears that etifoxine can act as an agonist, at least for αβ heteromers. It is possible, however, that modulatory effects

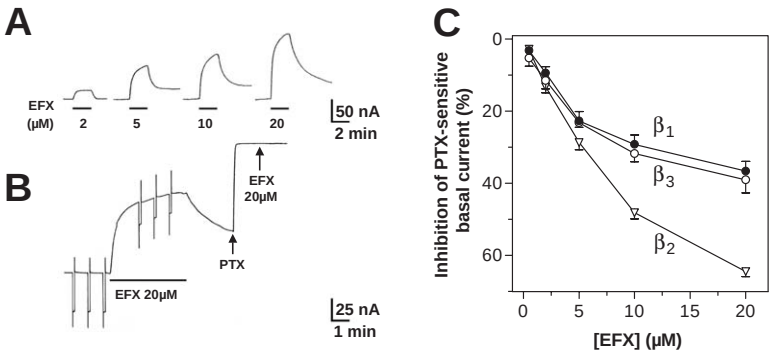


Fig. 1. Inhibitory effects of etifoxine (EFX) on the basal $Cl^-$ current recorded from *Xenopus* oocytes expressing murine $GABA_A$ receptor β subunits. (A) Sample traces showing the responses of a $\beta_2$-cDNA injected oocyte to increasing concentrations of etifoxine. The horizontal bars indicate the duration of drug application (2 min). Holding potential: −60 mV. (B) The basal conductance of homomeric $\beta_2$ receptors is reduced (from 4.3 mS to 2.2 mS) in the presence of 20 μM etifoxine, as indicated by the conductance pulses evoked by hyperpolarizing voltage steps (10 mV, 10 s) from the holding potential. After inhibition of the $Cl^-$ current by 100 μM picrotoxinin (PTX), etifoxine is inactive. (C) Concentration dependence of the etifoxine inhibition of the basal PTX-sensitive $Cl^-$ current recorded from homomeric β receptors. Data were normalized with respect to the amplitude of the PTX-sensitive control current (which was set to 100%) and are given as mean ± SEM ($n$ = 4–7). Mean values were found to be significantly different at high concentrations of etifoxine (10 and 20 μM) (ANOVA, $P < 0.05$).

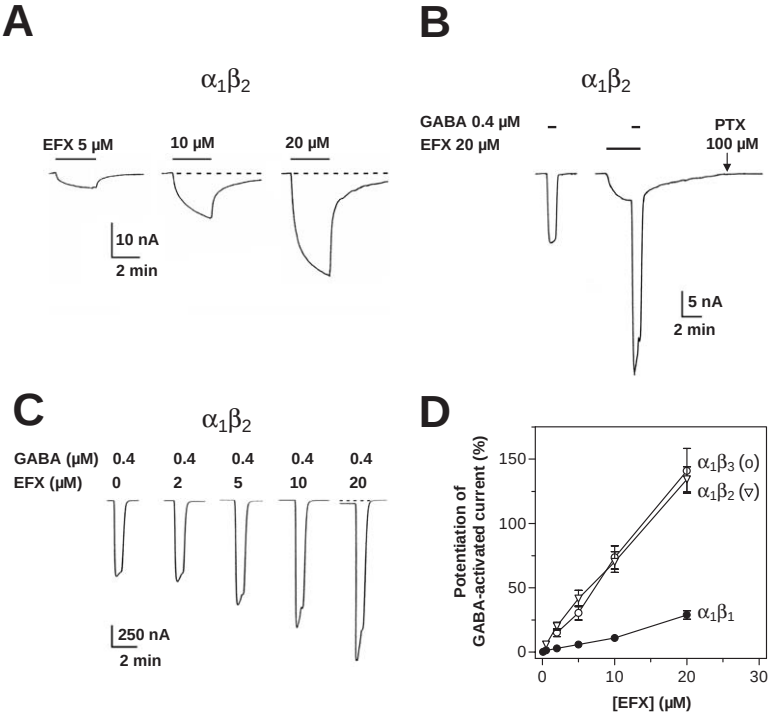


Fig. 2. Effects of etifoxine (EFX) on the basal $Cl^-$ current (A) and the GABA (EC10)-activated current (B–D) in oocytes expressing $\alpha_1\beta_x$ GABA receptors. (A) Currents recorded from an oocyte expressing $\alpha_1\beta_2$, in the presence of increasing concentrations of etifoxine. The horizontal bars indicate the duration of drug application. Etifoxine induces a concentration-dependent inward current instead of decreasing the basal current as observed for homomeric receptors. (B) Direct agonistic and potentiating effects of etifoxine in an oocyte expressing $\alpha_1\beta_2$ receptors and devoid of PTX-sensitive spontaneous current. (C) Sample traces illustrating the concentration-dependent potentiation of GABA (EC10)-activated currents by etifoxine, for an oocyte expressing $\alpha_1\beta_2$ receptors. Etifoxine was pre-applied for 2 min before being co-applied with GABA. (D) Etifoxine concentration–response relationship for the potentiation of GABA (EC10)-evoked currents for $\alpha_1\beta_1$, $\alpha_1\beta_2$ and $\alpha_1\beta_3$ receptors. Potentiation percentages were calculated with respect to the amplitude of the control. Data are given as mean ± SEM ($n$ = 4–11). Mean values were found to be significantly different at concentrations of etifoxine ≥2 μM (ANOVA, $P < 0.05$).

co-exist with direct agonist effects in oocytes showing spontaneous currents.

In the presence of etifoxine, the peak amplitude of currents evoked by EC10 concentrations of GABA was clearly higher than in control (Fig. 2B,C). This effect could not be explained by a simple addition of the etifoxine-activated current and the control GABA-activated current, but resulted from a genuine potentiation of GABA responses, (Fig. 2B). The replacement of the $\beta_1$ subunit by the $\beta_2$ or the $\beta_3$ subunits produced a 4–5-fold increase in the degree of potentiation induced by 20 μM etifoxine (Fig. 2D), thus revealing a stronger β-subunit dependence than with homomeric receptors.

The most important conclusion that emerges from these data is that a γ subunit is not required for etifoxine-potentiation of GABA receptor function. By contrast, it is well established that the high-affinity binding site of benzodiazepines involves the N-terminal domain of both α and γ subunits. Our observations are therefore in agreement with previous reports indicating that the effects of etifoxine are insensitive to flumazenil, an antagonist of the benzodiazepine site of GABA receptors (Verleye et al., 1999; Schlichter et al., 2000).

The $\beta_{2/3}$ preference of etifoxine is shared by other positive modulators of $GABA_A$ receptor function such as loreclezole (Wafford et al., 1994). For this anticonvulsant, the differential interaction with $GABA_A$Rs is determined by a single residue located within the M2 domain of the β subunit, an asparagine for $\beta_2$ and $\beta_3$ and a serine for $\beta_1$ (Wingrove et al., 1994). In order to determine whether etifoxine also acts via this residue (N-289 in $\beta_2$), we compared its modulatory effects on GABA (EC10) responses mediated by $\alpha_1\beta_2$ and $\alpha_1\beta_{2(N289S)}$ receptor channels. These experiments showed that etifoxine potentiation was not impaired by mutating N-289, as illustrated in Fig. 3 ($P > 0.05$, *t*-tests performed for 5, 10 and 20 μM etifoxine).

### 3.3. Heteromeric $\beta_x\gamma_{2s}$ receptors

In order to assess the absolute requirement of an α subunit for etifoxine potentiation of $GABA_A$ receptor function, subsequent experiments were carried out on recombinant receptors formed from the co-assembly of a β and a $\gamma_{2s}$ subunit. Numerous studies have shown that βγ subunit mixtures express with a reduced efficiency as compared to αβ combinations (see however Taylor et al., 1999 for $\beta_3\gamma_2$ receptors). In accordance with that view, we were able to obtain functional $\beta_1\gamma_{2s}$ and $\beta_2\gamma_{2s}$ receptors, but this required nuclear injection of large amounts of cDNAs. In addition, these receptors were expressed at low level, as evidenced by the small amplitude (≤250 nA) of their maximal GABA responses. At least some of the expressed receptors gated spontaneously, since small outward currents could be recorded in response to 100 μM PTX (not illustrated).

In spite of the erratic cell surface expression of βγ mixtures, we were able to observe that GABA-evoked currents were potentiated by etifoxine (Fig. 4). The overall characteristics of this potentiation, especially the higher efficiency of etifoxine on receptor isoforms containing a $\beta_2$ versus a $\beta_1$ subunit, were very similar to those described for αβ receptors. As the potentiation was not modified by replacement of the α subunit by a γ subunit, but was highly dependent on the nature of the β subunit, it appears likely that the binding site mediating these effects is located mainly within the β subunit.

### 3.4. Heteromeric $\alpha_1\beta_x\gamma_{2s}$ receptors

Most brain $GABA_A$ receptors are composed of α, β and γ subunits and the presence of these three types of subunits is required in recombinant receptors to mimic the full pharmacological profile of native receptors. In

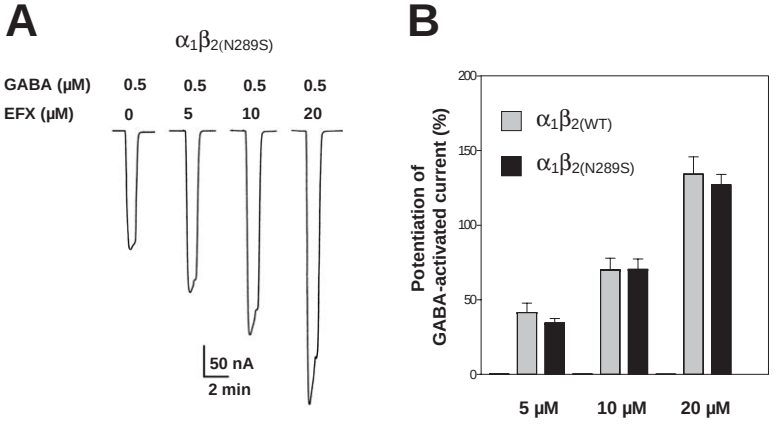


Fig. 3. Influence of the $\beta_{2(N289S)}$ mutation on the potentiating effects of etifoxine in oocytes expressing $\alpha_1\beta_2$ receptors. (A) Current traces showing the effects of etifoxine (5–20 μM) on the responses evoked by 0.5 μM GABA (EC10). (B) The potentiating effect of etifoxine at 5, 10 and 20 μM does not appear significantly different in oocytes expressing wild-type or mutant receptors (unpaired *t*-tests, $P > 0.05$). Data are given as mean ± SEM ($n = 6$).

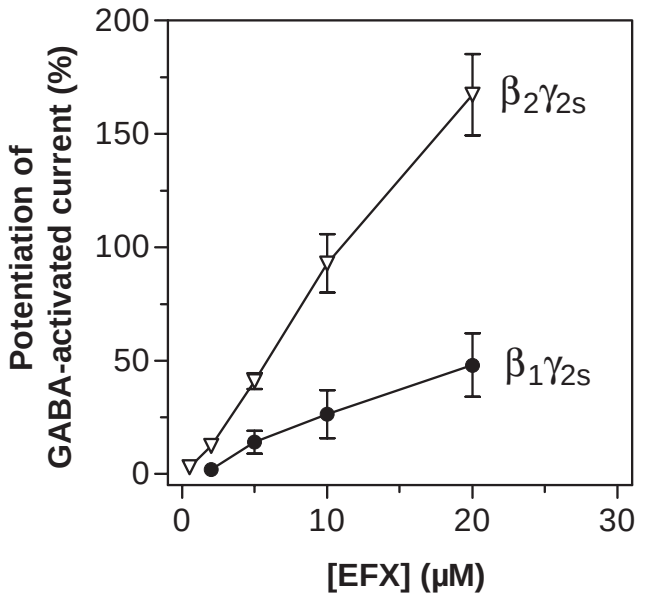


Fig. 4. Dose–response relationship for the potentiating effects of etifoxine on the GABA (EC10)-activated current in oocytes expressing $\beta_1\gamma_{2s}$ or $\beta_2\gamma_{2s}$ receptor channels. Mean values were found to be significantly different at concentrations of etifoxine ≥2 μM (unpaired *t*-test, $P < 0.05$).

order to determine whether the presence of a third subunit modifies the potentiating effects of etifoxine, we studied the potentiation of GABA responses mediated by $\alpha_1\beta_x\gamma_{2s}$ receptors.

Following co-injection of cDNAs coding for $\alpha_1$, $\beta_x$ and $\gamma_{2s}$ subunits, the PTX-sensitive spontaneous $Cl^-$ current was always small (<30–40 nA) when present, in contrast with the above observations on β or αβ receptors. In the presence of 10 μM $Zn^{2+}$, a cation that is known to block $\alpha_1\beta$ but not $\alpha_1\beta\gamma_2$ receptor channels (Draguhn et al., 1990), GABA-evoked currents were reduced by 16.2 ± 2.5% ($n = 6$) (Fig. 5A). This indicated that a small fraction of the total GABA currents was mediated by $\alpha_1\beta$ receptors coexisting with a larger population of αβγ heteromers. In order to eliminate the contribution of this fraction to the effects of etifoxine, subsequent experiments were carried out in the presence of 10 μM $Zn^{2+}$. Again, receptor isoforms containing a $\beta_2$ or a $\beta_3$ subunit were the most responsive to etifoxine (Fig. 5B). Statistical analysis of the magnitude of the GABA-potentiating effects of 20 μM etifoxine showed no significant difference between heterodimeric and heterotrimeric receptors, provided that their β subunit isoform was the same (ANOVA, $P > 0.05$, not illustrated).

The influence of GABA concentration on the ability of 20 μM etifoxine to potentiate $\alpha_1\beta_2\gamma_{2s}$ receptors was also investigated. In the presence of the modulator, the GABA dose–response curve was shifted to the left (Fig. 6) with a significant decrease in the EC50 value from 20.8 ± 1.2 μM to 7.5 ± 0.9 μM ($P < 0.05$), but there was no significant modification of the Hill slope (~1). In oocytes devoid of basal PTX-sensitive $Cl^-$ conductance, maximal GABA responses were the same as in controls, thus confirming previous data obtained from freshly dissociated dorsal horn neurons (Schlichter et al., 2000). However, in some oocytes exhibiting a basal PTX-sensitive current, the response to saturating concentrations of GABA was slightly decreased by etifoxine relatively to controls (not illustrated).

### 3.5. Heteromeric receptors containing an $\alpha_2$ subunit

While α subunits are not required for etifoxine activity, they might have a modulatory influence. In the spinal cord, etifoxine has a bell-shaped potentiating effect on $GABA_A$ currents recorded from dorsal horn neurons, maximum potentiation being observed at a concentration of 2 μM (Schlichter et al., 2000). As these neurons are primarily endowed with $\alpha_2$ and $\alpha_3$ subunits (Wisden et al., 1991; Bohlhalter et al., 1996), it is theoretically possible that the inhibitory effects observed at high doses of etifoxine might be linked to the presence of an α subunit different from $\alpha_1$. For this reason, comp-

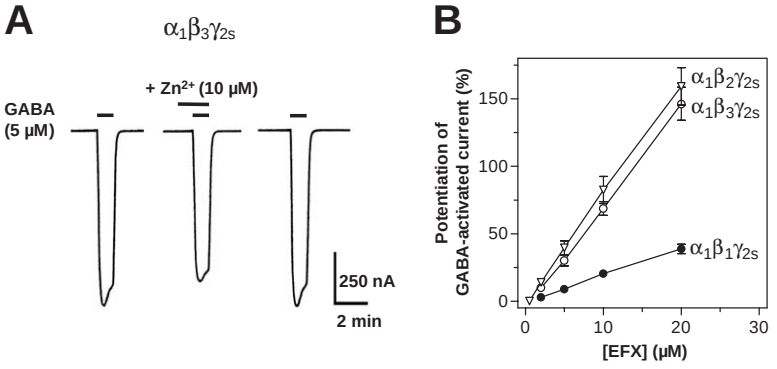


Fig. 5. Effects of etifoxine on $\alpha_1\beta_x\gamma_{2s}$ $GABA_A$Rs in the presence of $Zn^{2+}$. (A) Partial block of GABA-evoked currents by 10 μM $Zn^{2+}$ in an oocyte injected with equal ratios of $\alpha_1$, $\beta_3$ and $\gamma_{2s}$ $GABA_A$R cDNAs. (B) Dose-dependence of the modulatory action of etifoxine on $\alpha_1\beta_2\gamma_{2s}$ receptors, in the presence of $Zn^{2+}$ (to eliminate the contribution of $\alpha_1\beta_x$ receptors to potentiation). Potentiation percentages were calculated as in Fig. 2D. Mean values were found to be significantly different at concentrations of etifoxine ≥5 μM (ANOVA, $P < 0.05$).

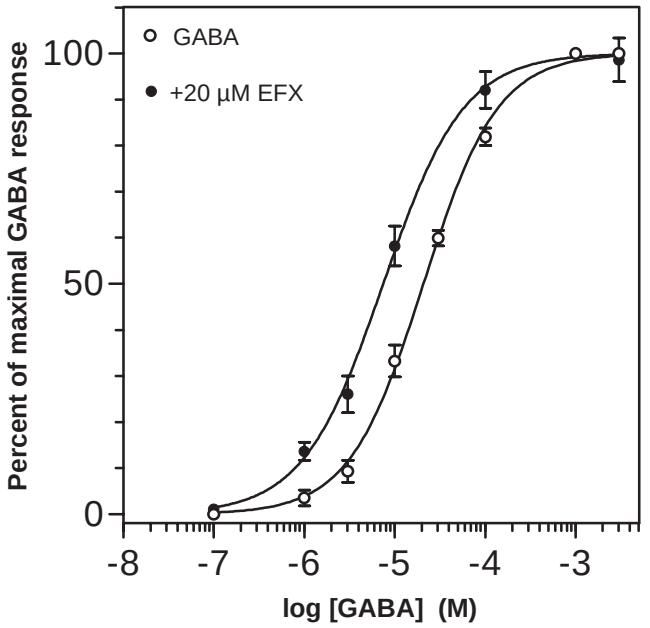


Fig. 6. Dose–response curves of GABA for $\alpha_1\beta_2\gamma_{2s}$ $GABA_A$Rs in the absence (empty symbols) and presence of 20 µM etifoxine (filled symbols). All peak current responses are normalized to the response evoked by 3 mM GABA. Data points are mean ± SEM of five independent measurements. Fitting the Hill equation to the data points gave an EC50 of 20.8 ± 1.2 µM in control and 7.5 ± 0.9 µM with etifoxine. These two values were found to be significantly different (unpaired *t*-test, $P < 0.05$). The decrease in the Hill coefficient (from 1.06 to 0.98) did not reach statistical significance.

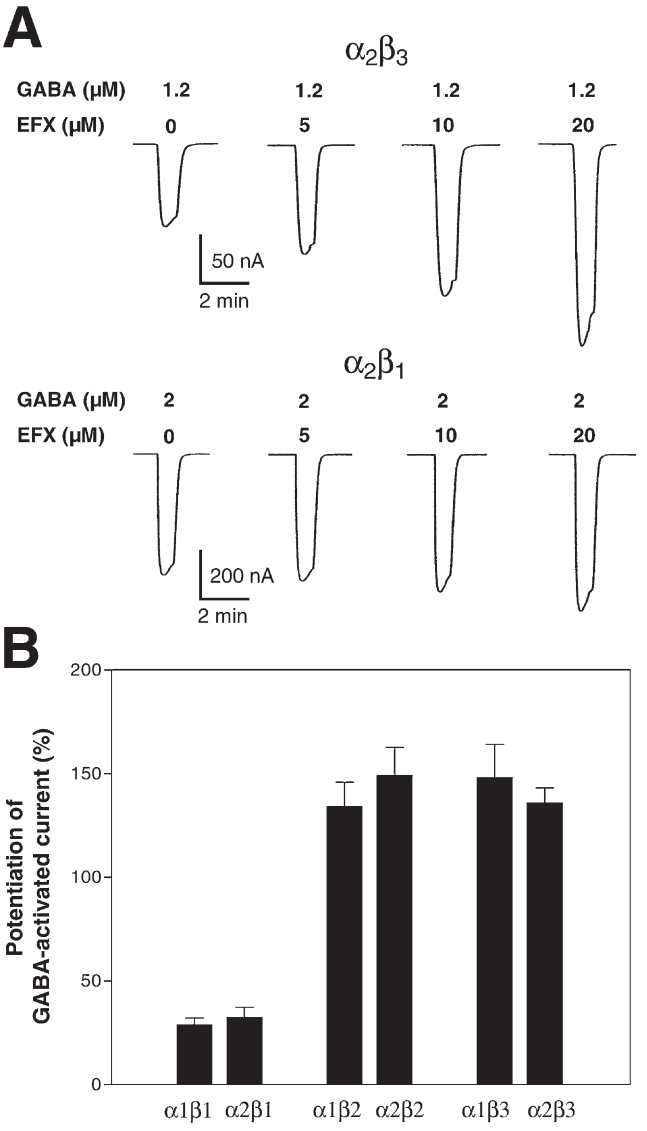


Fig. 7. Influence of the $\alpha_2$ subunit on the modulatory effects of etifoxine. (A) GABA (EC10)-evoked currents recorded from oocytes expressing $\alpha_2\beta_1$ and $\alpha_2\beta_3$ receptors, in the presence of increasing concentrations of etifoxine. (B) Histogram showing that the potentiation produced by 20 µM etifoxine is not modified by replacement of $\alpha_1$ by $\alpha_2$ in receptors containing different β subunits (unpaired *t*-tests, $P > 0.05$).

lementary experiments were carried out to compare the effects of etifoxine on $\alpha_1\beta_x$ and $\alpha_2\beta_x$ receptors. None of the $\alpha_2$-containing receptors displayed a reduced potentiation at high concentrations of etifoxine (illustrated for $\alpha_2\beta_1$ and $\alpha_2\beta_3$ in Fig. 7A). Another important result was that the $\beta_{2/3}$ preference of etifoxine was not significantly altered by replacement of $\alpha_1$ by $\alpha_2$ (Fig. 7B).

## 4. Discussion

### *4.1. Comparison with benzodiazepines, neurosteroids and barbiturates*

Our results demonstrate that the β subunit plays a major role in determining the potentiating effects of etifoxine on $GABA_A$ receptors. Whereas benzodiazepines require the simultaneous presence of an α and a γ subunit for activity at nanomolar concentrations (Sigel and Buhr, 1997; Korpi et al., 2002), etifoxine remains active in the absence of either of these two subunits. Therefore, it is clear that etifoxine does not target the high-affinity binding site of benzodiazepines which is located in the extracellular domain of the $GABA_A$R, at the αγ interface (Sigel and Buhr, 1997). This is in agreement with previous studies showing that flumazenil, an antagonist of central-type benzodiazepine sites, does not interfere with the behavioural effects of etifoxine (Verleye et al., 1999) or its electrophysiological effects on neuronal $GABA_A$Rs (Schlichter et al., 2000). Recently, however, it has been established that some benzodiazepines such as diazepam, but not flurazepam, produce a second component of potentiation at micromolar concentrations (Walters et al., 2000). Like the potentiation evoked by etifoxine, this component is independent of a γ subunit and insensitive to flumazenil. However, the micromolar activity of diazepam is observed only in the presence of very low GABA concentrations (<EC10), whereas etifoxine is effective for a broad range of agonist con-

centrations (Fig. 6), which may indicate that the two modulators act through distinct mechanisms.

Some of the functional properties of etifoxine are also shared by endogenous neurosteroids like allopregnanolone. These properties include anxiolytic and anticonvulsant effects, potentiation of GABA-stimulated $Cl^-$ currents or inhibition of TBPS binding (Lambert et al., 2001). Like etifoxine, allopregnanolone and other pregnane steroids are active on recombinant homomeric receptors composed of a β subunit and on heteromeric αβ and βγ receptors (Sanna et al., 1997; Maitra and Reynolds, 1998, 1999), demonstrating that the presence of an α or a γ subunit is not a prerequisite for neurosteroids to be active. It is very likely, however, that etifoxine and allopregnanolone bind to distinct recognition sites, as the two compounds produce only additive effects on TBPS binding or bicuculline-induced convulsions (Verleye et al., 2001). This view is further supported by our experiments showing that etifoxine is more active on ternary receptors containing $\beta_{2/3}$ subunits rather than the $\beta_1$ subunit, whereas pregnane steroids have similar effects on receptors containing $\beta_1$, $\beta_2$ or $\beta_3$ subunits (Maitra and Reynolds, 1998; Lambert et al., 2001; Belelli et al., 2002), indicating that their enhancing action is not influenced by the nature of the β subunit isoform. In addition, removal of the γ subunit does not significantly modify the potentiating effects of etifoxine, whereas the efficacy of allopregnanolone is considerably higher for $\alpha_1\beta_1$ than for $\alpha_1\beta_1\gamma_1$ receptors (Maitra and Reynolds, 1998).

The barbiturate pentobarbital also potentiates $GABA_A$ receptor function but its action differs from that of etifoxine in several aspects: (1) it generally increases the background conductance of homomeric β receptors, whereas etifoxine consistently inhibits these receptors, (2) it potentiates the maximal GABA current, (3) its effects on $\alpha_1\beta_x\gamma_2$ receptors are essentially unaffected by the β subunit isoform (Hadingham et al., 1993; Hill-Venning et al., 1997; Ghansah and Weiss, 2001). Together, these important differences suggest that etifoxine and barbiturates do not share the same mechanism of action.

### 4.2. Comparison with other positive modulators

The identity of the β subunit isoform has a crucial influence on the effects of other positive modulators of $GABA_A$ receptor function such as loreclezole (a broad-spectrum anticonvulsant), etomidate (an intravenous general anaesthetic) or mefenamic acid (MFA, a non-steroidal anti-inflammatory agent). All these compounds exhibit both GABA-mimetic and GABA-modulatory effects on $\beta_2$- and $\beta_3$-containing receptors, whereas their activity on $\beta_1$-containing receptors is comparatively very weak. This selectivity is conferred by an unique amino-acid residue, N-289 in human $\beta_2$ (N-290 in $\beta_3$, S-290 in $\beta_1$), located near the extracellular end of the second transmembrane domain (TM2) of the β subunit (Wafford et al., 1994; Belelli et al., 1997; Halliwell et al., 1999). Interestingly, this same residue is crucial for the micromolar effects of diazepam (Walters et al., 2000) and the β-carboline DMCM (Stevenson et al., 1995). It is not established whether these agents bind to the same site or, alternatively, share a common transduction mechanism. The slow onset and recovery times of their GABA mimetic action suggest that they do not access their recognition site via the channel lumen but, more likely, by entering the membrane phospholipid bilayer. In accordance with that view, the α subunit residue that is equivalent to $\beta_2$ N-289 has been proposed to face the phospholipid bilayer away from the channel lumen (Xu and Akabas, 1996).

Etifoxine shares some of the structural features of loreclezole, etomidate and MFA (Fig. 8) such as roughly similar molecular dimensions and the presence of two orthogonal hydrophobic rings in 3D-conformation. Many of its pharmacological characteristics also resemble those of these modulators, such as GABA-mimetic and GABA-potentiating effects at micromolar concentrations, selectivity for receptors containing the $\beta_2$ or the $\beta_3$ subunit over those containing the $\beta_1$ subunit, slow onset and recovery times of the GABA-mimetic action. Etifoxine is however characterized by two unique important properties: (1) it inhibits the basal activity of

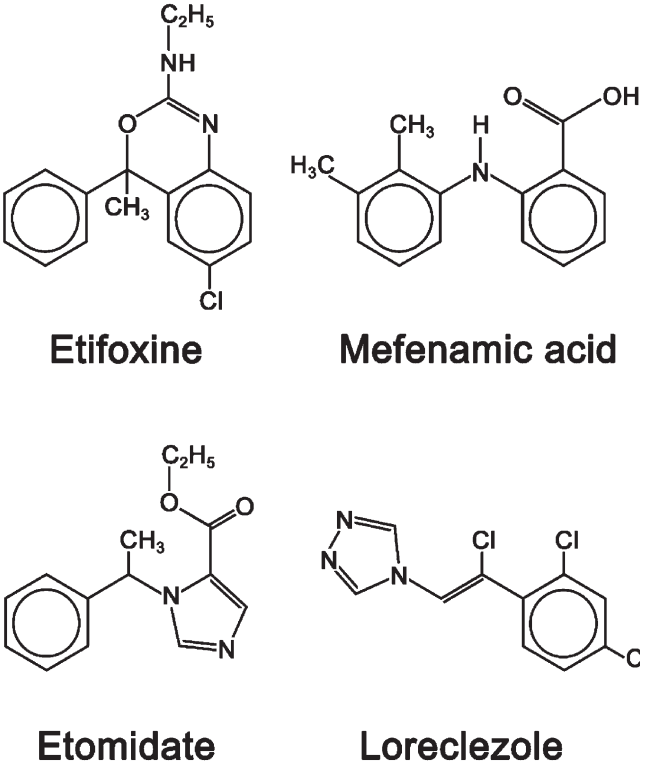


Fig. 8. Two-dimensional structures of etifoxine and other positive modulators of $GABA_A$Rs that are known to act via an asparagine in the TM2 domain of β subunits. The structures are represented in equivalent orientations with respect to their hydrophobic regions.

all homomeric β receptors, (2) replacement of $\beta_2$ N-289 by a serine does not impair its modulatory action. It seems premature, however, to speculate that etifoxine acts via a novel allosteric modulatory site on the $GABA_A$ receptor. Further work including competitive binding assays and point mutation experiments is required to clarify this issue.

### *4.3. Comparative effects of etifoxine on native and recombinant receptors*

Our observation that etifoxine modulates the function of recombinant $GABA_A$ receptors is in agreement with previous studies on spinal cord and hypothalamic cultured neurons (Schlichter et al., 2000). However, native and expressed receptors differ in their pattern of modulation in at least two aspects. Firstly, the tonic $GABA_AR$ $Cl^-$ current recorded from hypothalamic neurons is consistently potentiated by etifoxine, whereas the background $Cl^-$ current recorded from oocytes was either inhibited or enhanced, depending on whether a β subunit was expressed alone or in combination with other subunits. This certainly means that hypothalamic neurons do not express homomeric β receptors, as also indicated by their sensitivity to the blocking effects of bicuculline (Schlichter et al., 2000), which contrasts with the inability of this antagonist to block rodent or human β homomers (Sanna et al., 1995; Krishek et al., 1996; Cestari et al., 1996; Wooltorton et al., 1997). Secondly, in dorsal horn neurons dissociated from laminae I–II, etifoxine has a biphasic, bell-shaped potentiating effect, whereas in oocytes its action increases regularly with concentration, at least to 20 μM. The in vivo decline of potentiation at high concentrations might reflect binding of etifoxine to a low-affinity inhibitory site, resulting either in channel blockade or enhanced desensitisation, as shown for loreclezole (Donnelly and McDonald, 1996; Fisher et al., 2000). Since dorsal horn neurons (in laminae I–II) are primarily endowed with $\alpha_2$ and $\alpha_3$ subunits (Wisden et al., 1991; Bohlhalter et al., 1996), it was theoretically possible that the inhibitory effects observed at high doses of etifoxine might be correlated with the presence of an $\alpha_2$ subunit isoform. However, in our experiments, $\alpha_2$-containing receptors did not show any decline of the potentiation at high concentrations of etifoxine (Fig. 7). Therefore, it is more likely that the differential activity of etifoxine on neurons and oocytes originates from differences in experimental procedures, especially differences in the kinetics of drug application. In the experiments of Schlichter et al. (2000), the small size of neurons combined with the use of a concentration–clamp technique for drug application, allowed a rapid exposure of membrane receptors to GABA and, therefore, a minimal degree of desensitisation before the peak current. On the contrary, the very large size of oocytes and application of drugs via the perfusate are known to result in a large amount of desensitisation during the ascending phase of currents, which in turn affects Hill coefficients, drug potencies and even the shape of dose–response curves, as shown for ethanol and pentobarbital (Harris et al., 1997).

In conclusion, the anxiolytic agent etifoxine acts preferentially on $GABA_A$ receptors containing the $\beta_2$ or the $\beta_3$ subunit. Its potentiating effects should therefore be maximal in brain areas where these subunits predominate over the $\beta_1$ isoform. Further studies will be required to determine what amino-acid residues underlie the preference of etifoxine for $\beta_{2/3}$-containing receptors.

## Acknowledgements

This work was supported by Biocodex Laboratories (Compiègne, France). We would like to thank Pr. Stephen J. Moss (University College, London) for the $\alpha_1$, $\beta_{1-3}$ and $\gamma_{2s}$ subunits, Pr. Hartmut Lüddens (University of Mainz, Germany) for the $\alpha_2$ subunit and Pr. Neil L. Harrison (Cornell University, New York, USA) for the $\beta_{2(N289S)}$ subunit. We would also like to thank Pr. Rémy Schlichter (University of Strasbourg, France), Pr. Bruno Lapied (University of Angers, France) for helpful discussions, and Pr. René H. Levy (University of Washington, Seattle, USA) for carefully reading the manuscript.

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