Pergamon

Neuropharmacology 39 (2000) 1523–1535

NEURO PHARMACOLOGY

www.elsevier.com/locate/neuropharm

Modulation of GABAergic synaptic transmission by the non-benzodiazepine anxiolytic etifoxine

Rémy Schlichter [a,*], Volodymyr Rybalchenko [a], Pierrick Poisbeau [a], Marc Verleye [b], Jean-Marie Gillardin [b]

[a] *Laboratoire de Neurophysiologie cellulaire et intégrée, UMR 7519 CNRS, Université Louis Pasteur, 21 rue René Descartes, 67084 Strasbourg Cedex, France*

[b] *Laboratoires Biocodex, Centre de recherche, 60200 Compiègne, France*

Accepted 10 November 1999

Abstract

We have investigated the effects of 2-ethylamino-6-chloro-4-methyl-4-phenyl-4*H*-3,1-benzoxazine hydrochloride (etifoxine) on $GABA_A$ receptor function. Etifoxine displaced [^{35}S]TBPS (*t*-butylbicyclophosphorothionate) from $GABA_A$ receptors of rat cortical membranes with an IC_{50} of 6.7±0.8 μM and [^{3}H]PK11195 from peripheral (mitochondrial)-type benzodiazepine receptors (PBRs) of rat heart homogenates with an IC_{50} of 27.3±1.0 μM. Etifoxine displayed anxiolytic properties in an anticonflict test in rats, and potentiated $GABA_A$ receptor-mediated membrane currents elicited by submaximal (5–10 μM) but not saturating (0.5 mM) concentrations of GABA in cultured rat hypothalamic and spinal cord dorsal horn neurones. In hypothalamic cultures, etifoxine induced a dose-dependent inward current for concentrations >1 μM which reflected the post-synaptic potentiation of a small (~20 pA) tonic and bicuculline-sensitive $GABA_A$ receptor-gated Cl^- current. Etifoxine also increased the frequency of spontaneous and miniature GABAergic inhibitory post-synaptic currents without changing their amplitude and kinetic characteristics. Both effects of etifoxine were insensitive to flumazenil (10 μM), an antagonist of central-type benzodiazepine sites present at $GABA_A$ receptors, but were partly inhibited by PK11195 (10 μM) an antagonist of PBRs which control the synthesis of neurosteroids. Our results indicate that etifoxine potentiates $GABA_A$ receptor-function by a direct allosteric effect and by an indirect mechanism involving the activation of PBRs.

Keywords: Peripheral benzodiazepine receptor (PBR); Inhibitory post-synaptic current (IPSC); Anxiolytic; Neurosteroid; PK11195

1. Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system (CNS) of vertebrates and activates both ionotropic ($GABA_A$) and G-protein-coupled ($GABA_B$) receptors. $GABA_A$ receptors ($GABA_A$Rs) are ligand-gated Cl^- channels formed by the pentameric assembly of distinct subunits and mediate the fast component of GABAergic synaptic transmission (Macdonald and Olsen, 1994; Sieghart, 1995; Mehta and Ticku, 1999). To date, 15 different $GABA_A$R subunits have been identified. Among these, α and β subunits are of fundamental importance for the formation of the Cl^- channel and the binding of the agonist, whereas other subunits confer on $GABA_A$Rs a sensitivity/insensitivity to endogenous and/or exogenous allosteric modulators (Macdonald and Olsen, 1994; Sieghart, 1995; Mehta and Ticku, 1999). For example, the presence of γ subunits confers $GABA_A$R sensitivity to benzodiazepines (Pritchett et al., 1989; Sigel and Buhr, 1997; Mehta and Ticku, 1999), whereas the presence of δ (Zhu et al., 1996) or ε (Davies et al., 1997) subunits renders $GABA_A$Rs insensitive to the modulatory action of neuroactive steroids, i.e. potent allosteric modulators of $GABA_A$Rs which are synthesised within the CNS by neurones and glial cells (Majewska, 1992; Robel and Beaulieu, 1994; Lambert et al., 1995; Mensah-Nyagan et al., 1999).

Both benzodiazepines (Defazio and Hablitz, 1998; Perrais and Ropert, 1999) and neurosteroids (Harrison et al., 1987; Poisbeau et al., 1997) were shown to potentiate

* Corresponding author. Tel.: +33-3-88-41-60-18; fax: +33-3-88-61-33-47.

E-mail address: schlichter@neurochem.u-strasbg.fr (R. Schlichter).

0028-3908/00/$ - see front matter
PII: S0028-3908(99)00253-1

GABAergic synaptic transmission, a phenomenon which probably largely contributes to the anxiolytic and anticonvulsant properties of these positive $GABA_AR$ modulators. Yet, benzodiazepines, which are of wide clinical use, display a series of side-effects including sedation, motor and memory impairment and exhibit tolerance and rebound effects during withdrawal after prolonged administration (Sigel and Buhr, 1997; Gasior et al., 1999; Mehta and Ticku, 1999). Interestingly, natural or synthetic neurosteroids seem devoid of major side-effects, including tolerance and dependence, but are just beginning to be used in clinical practice (Gasior et al., 1999). However, many synthetic molecules, although not structurally related to benzodiazepines, neurosteroids or barbiturates, also possess efficient anxiolytic and/or anticonvulsant properties. For example, etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4*H*-3,1-benzoxazine hydrochloride; Stresam®) was shown to have anticonvulsant and anxiolytic properties in rodents (Boissier et al., 1972) suggesting an interaction with the GABAergic system (Kruse and Kuch, 1985) and a recent study in humans indicates the interest of using etifoxine in the treatment of adjustment disorder with anxiety (Servant et al., 1998). We have used radioligand binding to characterise the interaction of etifoxine with $GABA_A$ receptors from rat cortical membranes and peripheral (mitochondrial) benzodiazepine receptors from rat heart tissue homogenates. We also assessed the anxiolytic properties of etifoxine using Vogel's conflict test (Vogel et al., 1971). In addition, a major aim of our study was to characterise the effects of etifoxine on $GABA_AR$-mediated membrane currents and inhibitory post-synaptic currents (IPSCs). For this purpose, we used cultures of foetal rat hypothalamic neurones in which we had previously characterised $GABA_A$ receptor-mediated synaptic transmission and its modulation by neurosteroids (Poisbeau et al., 1997). We also used dissociated laminae I–II rat dorsal horn (DH) spinal cord neurones which posses functional $GABA_A$ receptors and permit, unlike freshly dissociated hypothalamic neurones which are too fragile, fast agonist application over the total surface of the cell by means of the concentration-clamp technique (Rybalchenko and Schlichter, 1999). Our results indicate that (1) etifoxine is a positive allosteric modulator, binding to $GABA_ARs$ at a site distinct from that of benzodiazepines and (2) part of the effect of etifoxine seems to involve the activation of peripheral (mitochondrial)-type benzodiazepine receptors which are not located on $GABA_A$ receptors.

2. Methods

2.1. Binding experiments

2.1.1. Preparation of membranes

Binding experiments were performed as described previously (Table 1).

Briefly, after decapitation, the brain and/or the heart of male Sprague-Dawley rats (150–200 g) were removed after careful dissection and the cortex or the ventricular heart tissue was homogenised in an appropriate buffer. Ventricular heart tissue homogenate is a classically used preparation for assessing the binding of substances to peripheral benzodiazepine receptors (Le Fur et al., 1983). Membranes were incubated under the conditions described in Table 1. Following incubation, cortical membranes or homogenates of ventricular heart tissue were rapidly filtered under vacuum through GF/B or GF/C glass fibre (Whatman or Packard) and washed several times with an ice-cold buffer using a cell harvester (Brandel or Packard). The filters were then immersed in vials containing the scintillation cocktail (formula 989, N.E.N., Le Blanc-Mesnil, France) and bound radioactivity was determined using a Beckman LS6000 liquid scintillation counter.

2.1.2. Drugs

Stock solutions of etifoxine (Biocodex-France) (100 mM) were made up in dimethylsulphoxide (DMSO) and diluted before use in distilled water (final concentration of DMSO$\leq$0.1%). Radiolabelled ligands were obtained from N.E.N. All other substances were obtained from Sigma or Research Biochemicals Inc. (RBI, Bioblock Scientific, Illkirch, France).

2.1.3. Data analysis and expression of results

Specific radioligand binding in each assay was defined as the difference between total binding and non-specific binding determined in the presence of an excess of unlabelled competitor (details are given in Table 1). Each independent experiment ($n\geq2$) was replicated three times. Data are expressed as the mean±SEM.

The concentration of compound that produced half-maximal inhibition (IC_{50}) of specific control radiolabelled ligand binding was determined by non-linear regression analysis of the competition curve (Sigmaplot®; V4.1; Jandel Scientific). This parameter was obtained by Hill equation curve fitting.

2.2. Behavioural testing

2.2.1. Animals

Male Wistar rats weighing between 160 and 180 g were housed in standard polypropylene cages (10 per cage) in a room at constant temperature (22±2°C) with a 12 hr light/dark cycle and with controlled humidity (50±20%). Food (UAR-A04) and tapwater were freely available. Animals were habituated for a minimum of 4 days prior to experimentation. The experiments were conducted in accordance with the European recommen-

Table 1
Methodological procedures for in vitro binding assays[a]

Assay	Tissue (rat)	Specific binding (nM)	Non-specific binding (μM)	Incubation	Reference
Receptor					
$GABA_A$	Cerebral cortex	[^{3}H]Muscimol (2.5)	Muscimol (10)	10 min/4°C	Snodgrass (1978)
BZD central	Cerebral cortex	[^{3}H]Flunitrazepam (0.4)	Diazepam (3)	60 min/4°C	Speth et al. (1979)
BZD peripheral	Heart	[^{3}H]PK 11195 (0.2)	PK 11195 (10)	15 min/25°C	Le Fur et al. (1983)
5-$HT1_A$	Cerebral cortex	[^{3}H]8-OH-DPAT (0.5)	8-OH-DPAT (10)	30 min/25°C	Hall et al. (1985)
5-HT2	Cerebral cortex	[^{3}H]Ketanserine (0.5)	Ketanserine (1)	40min/25°C	Leysen et al. (1982)
Uptake site					
GABA	Cerebral cortex	[^{3}H]GABA (10) (+10 μM isoguvacine) (+10 μM baclofen)	GABA (1000)	30 min/25°C	Shank et al. (1990)
NE	Cerebral cortex	[^{3}H]Nisoxetine (1)	Desipramine (1)	240 min/4°C	Tejani-Butt (1992)
Chloride channel site					
	Cerebral cortex	[^{35}S]TBPS (4)	Picrotoxinine (20)	90 min/25°C	Lewin et al. (1989)

[a] Abbreviations: GABA, γ-amino-butyric acid; BZD, benzodiazepine; NE, norepinephrine; TBPS, *t*-butylbicyclophosphorothionate; 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin.

dations governing the protection of animals used for experimental purposes and were approved by the local animal care committee.

2.2.2. Conflict test

Vogel's conflict test (Vogel et al., 1971; Gasior et al., 1999) was used to assess the effect of etifoxine on anxiety. Rats were deprived of water for 48 hr prior to the test session. Each animal was then placed in a conflict test box (Letica-model 8600) for 5 min and was allowed to explore and to lick 40 times the drinking spout before being removed from the box. Only rats licking during this session were used. Six hours after this adaptation session, the second experimental session, lasting 3 min, started automatically when the rat completed 20 licks and received the first mild electric shock (0.5 mA, 1 sec). After 20 unpunished licks, subsequent licking was punished.

The number of shocks accepted throughout the 3 min experimental session and the time elapsed before the first shock were recorded.

2.2.3. Drug treatment

Etifoxine was administered intraperitoneally (ip) 35 min prior to the experimental session at doses of 12.5, 25 and 50 mg/kg. The control animals received an equivalent volume (0.5 ml/100 g) of vehicle (1% Tween in 0.9% NaCl solution).

2.2.4. Statistical analysis

The results are expressed as mean±SEM. Statistical analysis was performed with the non-parametric Kruskall Wallis test followed by Dunn's test to localise the differences between control and treated animals (Sigmastat®; V.2.0; Jandel Scientific). The accepted level of significance was $P<0.05$.

2.2.5. Cell culture and electrophysiology

2.2.5.1. Dorsal horn (DH) neurones After decapitation under deep diethyl-ether anaesthesia, the spinal cord from 9–14-day-old Wistar rats was removed and transferred into cold (4°C) sucrose buffer (in mM: 261 sucrose, 2 KCl, 1 $MgCl_2$, 1.25 KH_2PO_4, 10 glucose, 26 $NaHCO_3$). Punches of tissue corresponding to laminae I–II were dissociated enzymatically for 55 min at 37°C in Ca^{2+}–Mg^{2+}-free Earle's balanced salts solution (Gibco) containing papain (21 IU/ml) and L-cysteine (0.1 mM) and bubbled with 5% CO_2 and 95% O_2. After mechanical dissociation, the cells were suspended in culture medium composed of MEM alpha (Gibco), fetal calf serum (FCS; Gibco, 7% vol/vol) and penicillin–streptomycin (Gibco, 50 IU/ml each), and seeded onto 35 mm culture dishes (Costar, Corning). Cells were kept at 37°C in a water-saturated atmosphere containing 5% CO_2 and 95% air until use (6–20 hr).

2.2.5.2. Hypothalamic neurone cultures Cultures of Wistar rat hypothalamic neurones were prepared as described previously (Poisbeau et al., 1996). The diencephalic area corresponding to hypothalamus was isolated from 15 or 16-day-old rat foetuses and enzymatically dissociated with trypsin–EDTA (0.05%–0.02%, Gibco) for 7 min at 37°C under constant shaking. The enzymatic dissociation was stopped by adding 20% FCS and followed by centrifugation for 3 min at 180**g**. Mechanical dissociation was performed in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) containing

20% FCS with fire-polished Pasteur pipettes. After centrifugation (7 min at 180**g**), the pellet containing the dissociated cells was resuspended in serum-free medium according to the method of Bottenstein and Sato (1979). Cultures were maintained in a water-saturated atmosphere (95% air, 5% CO_2) at 37°C until use for electrophysiological experiments (7–13 days).

2.2.6. Electrophysiological recordings and application of solutions

All recordings were performed under voltage-clamp in the whole-cell configuration of the patch-clamp technique and at room temperature (20–22°C). Two types of agonist application methods were used: (1) the concentration-clamp technique (Rybalchenko and Schlichter, 1999) which was employed with DH neurones and (2) the U-tube application technique (Fenwick et al., 1982) combined with general bath perfusion for hypothalamic neurones. Different techniques for agonist application were used because freshly dissociated hypothalamic neurones were too fragile for the fast concentration-clamp technique.

2.2.6.1. Concentration-clamp technique DH neurones were voltage-clamped at −40 mV in the whole-cell configuration. Agonist application was performed with a modified version of the concentration-clamp technique which allowed solution exchange over the total surface of the cell within 6–8 ms (Rybalchenko and Schlichter, 1999). Moreover, under this recording configuration, the cell attached to the tip of the pipette was completely isolated, i.e. physically separated, from neighbouring cells (neurones or glial cells). The external solution contained (in mM): NaCl 140, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, glucose 5 and HEPES 10 (pH 7.4 with TrisOH). Recording pipettes (2–4 MΩ resistance) were filled with a Ca^{2+}-free intracellular solution containing (in mM): CsCl 140, EGTA 10, MgATP 2, NaGTP 0.2, HEPES 10 (pH 7.45 with TrisOH). Membrane currents were recorded with an EPC-7 amplifier (List Medical) and digitised at 2 kHz with a TL-1 interface (Axon Instruments) after being filtered at 1 kHz. We verified that filtering at 2 kHz and digitising at 4 kHz gave similar results.

2.2.6.2. U-tube application and bath perfusion Patch-clamp recordings from hypothalamic neurones were made in the whole-cell configuration using an Axopatch 200A amplifier (Axon instruments, USA) and low-resistance (3–4 MΩ) electrodes. Membrane currents were filtered at 5 kHz and stored on videotape (resolution, 20 kHz). The standard external medium contained (in mM) NaCl 135, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 5, glucose 10 (pH 7.4, adjusted with NaOH). Pipettes were filled with an intracellular solution containing (in mM) CsCl 125, $CaCl_2$ 5, HEPES 10, EGTA 10, Mg-ATP 2 (pH 7.3 with CsOH). The estimated intracellular free calcium concentration was 10^{-7} M and the equilibrium potential for chloride ions (E_{Cl}) was −2 mV. The holding potential was −60 mV.

GABA was diluted in external solution and applied locally using a "U-tube" (Fenwick et al., 1982). All other substances were applied by bath perfusion at a rate of 3 ml/min for a total bath volume of 1.5 ml. When GABA was applied in the presence of a given substance in the extracellular medium, the same concentration of the substance was added to the GABA solution which was applied locally by means of the U-tube.

2.2.7. Solutions

GABA (Sigma, France), bicuculline (Sigma, France), and tetrodotoxin (TTX, Latoxan, France) were prepared as 1000 times concentrated stock solutions in distilled water and stored at −20°C. PK11195 (Sigma, France) was prepared at a concentration of 10 mM in ethanol and stored at 4°C. Etifoxine (Biocodex, France) and flumazenil (a kind gift of Roche, Basel, Switzerland) were prepared as 1000 times concentrated stock solutions in DMSO just before the experiment and diluted to their final concentration in extracellular medium. At the same dilution, DMSO and ethanol alone had no effect on $GABA_A$ receptor currents or GABAergic IPSCs.

2.2.8. Analysis of electrophysiological data

Electrophysiological data were stored in digital form on video tape or on the hard disk of a personal computer. Analysis of GABA-induced whole-cell currents and statistical tests were performed with Origin (Microcal software). Synaptic currents were analysed with the program SYNAPSE, written, developed and kindly supplied by Dr Yves De Konninck (McGill University, Montreal, Canada). Off-line analysis was performed on synaptic currents after filtering at 2 kHz and digitisation at 4 kHz of the current traces.

All statistical results concerning electrophysiological data are given as mean±standard deviation (s.d).

3. Results

3.1. Behavioural effects of etifoxine

Etifoxine dose-dependently increased punished drinking with a statistical significant effect at doses of 25 and 50 mg/kg (Table 2). This effect of etifoxine was consistent with anxiolysis. Etifoxine failed to produce any significant effect on spontaneous water drinking or shock thresholds even at the highest dose tested (50 mg/kg; data not shown). The latter point also suggested that etifoxine apparently had no analgesic effect.

The time elapsed to find again the drinking spout (first shock latency) did not differ between etifoxine-treated

Table 2
Effects of etifoxine in Vogel's conflict test in the rat[a]

Treatment and dose (mg/kg)	*N*	Number of shocks accepted/3 min (mean±SEM)	1st shock latency (sec) (mean±SEM)
Controls	17	2.8±0.8	54.7±9.2
Etifoxine (12.5)	10	5.7±1.3	63.7±14.8
Etifoxine (25)	10	11.1±2.6*	46.1±10.8
Etifoxine (50)	10	20.4±3.2*	50.4±8.2

[a] * $p<0.05$ compared to controls. *N*=number of animals used.

and control animals (Table 2). This result indicated that etifoxine did not impair motor activity.

3.2. Binding experiments

The results of binding experiments are summarised in Table 3. Etifoxine displaced [^{35}S]TBPS (*t*-butylbicyclophosphorothionate) from rat brain cortical membranes with an IC_{50} of 6.7±0.8 μM. This compound also inhibited specific binding of [^{3}H]PK11195, a ligand of peripheral (mitochondrial) benzodiazepine receptors (Benavides et al., 1983) on rat heart tissue homogenates, with an IC_{50} of 27.3±1.0 μM. Etifoxine displayed a moderate affinity for norepinephrine (NE) and GABA uptake sites but exhibited no significant binding to $GABA_A$ receptor sites, central benzodiazepine receptor sites or 5-HT_{1A} and 5-HT_2 serotonin receptor sites, at concentrations up to 100 μM.

3.3. Effect of etifoxine on GABA-induced whole-cell currents

We tested the effect of etifoxine on $GABA_A$ receptor-mediated Cl^- currents in spinal cord and hypothalamic neurones at concentrations up to 30 μM. At higher concentrations etifoxine displayed limited solubility in physiological extracellular solutions, and could therefore not be used reliably at concentrations >30 μM.

Table 3
Affinity of etifoxine for different binding sites in membrane preparations[a]

Binding	Etifoxine IC_{50} (μM)
Ligand/binding site	
[^{3}H]Muscimol/$GABA_A$	>100
[^{3}H]Flunitrazepam/$BZD_{central}$	>100
[^{3}H]PK11195/$BZD_{peripheral}$[b]	27.3±1.0
[^{3}H]8-OH-DPAT/5-$HT1_A$	>100
[^{3}H]Ketanserine/5-HT2	>100
Ligand/uptake site	
[^{3}H]GABA/GABA uptake	78
[^{3}H]Nisoxetine/NE uptake	39
[^{35}S]TBPS/Chloride channel[b]	6.7±0.8

[a] Abbreviations: BZD, benzodiazepine; NE, norepinephrine; TBPS, *t*-butylbicyclophosphorothionate; 5-HT, 5-hydroxytryptamine (serotonin); 8-OH-DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin.

[b] For these binding sites, three independent experiments were carried out in triplicate. The data represent the mean±SEM. [^{3}H]PK11195 binding was performed on heart membrane homogenates.

3.3.1. DH neurones

Superficial DH neurones express functional $GABA_A$ receptors which underlie synaptic inhibition in the spinal nociceptive system (Malcangio and Bowery, 1996). Application of etifoxine alone did not induce any membrane current in isolated DH neurones at any concentration (up to 30 μM) tested (*n*=14). In contrast, etifoxine potentiated $GABA_A$ responses to submaximal GABA concentrations (10 μM, *n*=14) while having no effect on saturating concentrations of GABA (0.5 mM, *n*=6). On the basis of the effect of etifoxine, the responsive neurones were classified into two categories which we termed type I and type II (Fig. 1). Type I neurones displayed substantial inward currents (mean amplitude: −365.7±79.9 pA, *n*=6) in response to 10 μM GABA, a concentration which represented approximately the EC_{50} value for the GABA dose–response relationship of these neurones. Etifoxine had a bell-shaped potentiating effect on $GABA_A$ currents in type I neurones (Fig. 1a and b). The threshold was around 0.1 μM and the maximal effect was observed at a concentration of 2 μM. At higher concentrations (10–20 μM) this potentiating effect decreased again, but an inhibition of the control current was not observed. Type II neurones (Fig. 1c and d) were characterised by very small responses (mean amplitude: −10.4±5.4 pA, *n*=8) to 10 μM GABA, which represented a just supra-threshold concentration on the GABA dose–response relationship of these neurones. These currents were maximally potentiated by 2 μM etifoxine and the threshold concentration was around 0.1 μM. The bell-shaped character of the potentiating effect of etifoxine was less apparent in these neurones (Fig. 1d) than in type I neurones.

3.3.2. Hypothalamic neurones

Freshly dissociated embryonic hypothalamic neurones were too fragile to be studied with the concentration-clamp technique. Therefore, GABA was applied by means of a U-tube (see Methods) at a concentration of 5 μM which corresponded to a just supra-threshold concentration on the GABA dose–response relationship of these neurones. Etifoxine (10 μM) potentiated the submaximal responses to 5 μM GABA by 62±14% (*n*=10), but had no effect on maximal $GABA_A$ currents in response to 0.5 mM GABA (*n*=5). However, in contrast to isolated DH neurones, we noticed that bath application of etifoxine alone induced (modulated) a slow inward membrane current which we decided to study in more detail (see below).

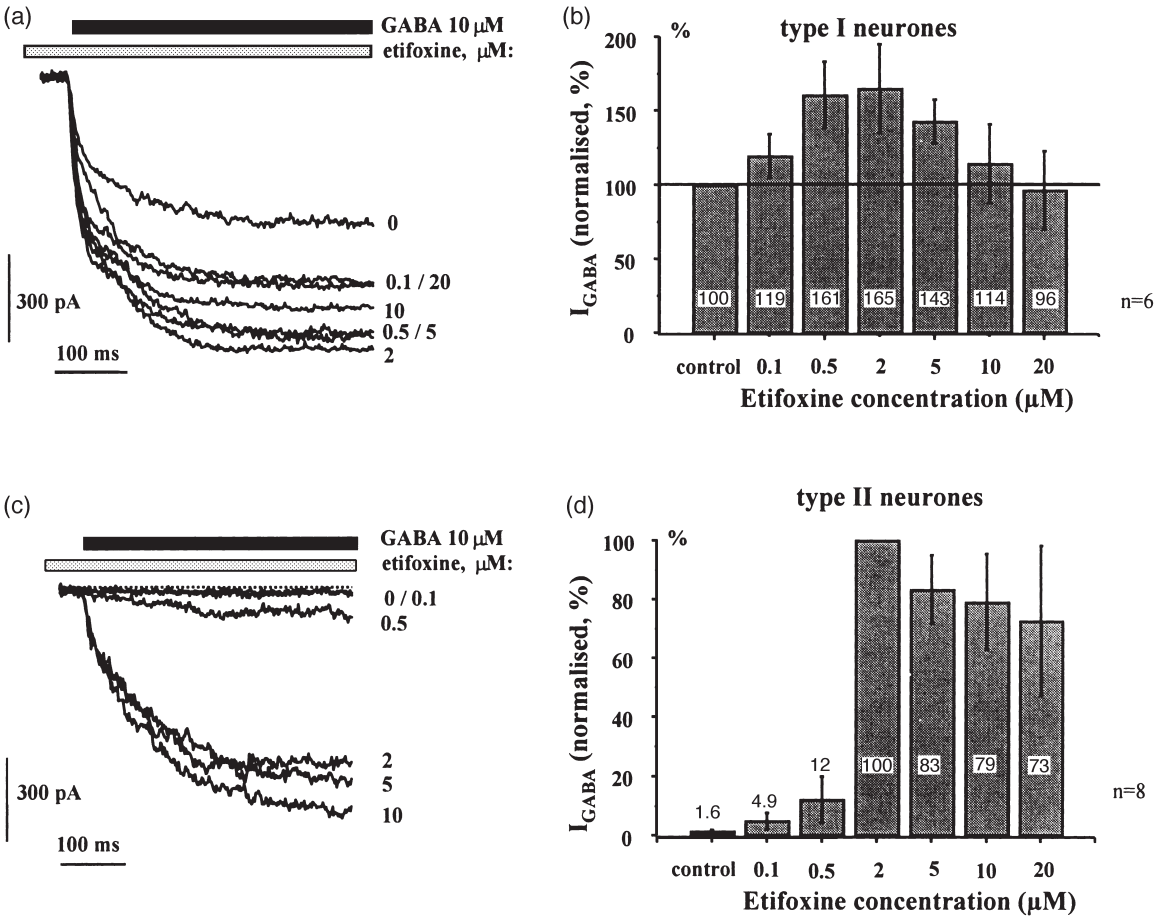

Fig. 1. Effect of various concentrations of etifoxine on submaximal $GABA_A$R-gated currents evoked by 10 μM GABA in freshly dissociated spinal cord dorsal horn (DH) neurones. The substances were applied to the isolated neurones with the concentration-clamp technique. (a) GABA (10 μM) was applied for the duration indicated by the horizontal black bar either in the absence (trace labelled 0) or in the presence of increasing concentrations of etifoxine. The labels on the right of the traces indicate the concentration of etifoxine used (in μM). Etifoxine alone was preapplied for 40 sec before being coapplied with GABA. In this neurone GABA alone elicited a large (>300 pA) inward current and the potentiation of the current was maximal for 2 μM etifoxine. These neurones were designated as type I neurones. (b) Potentiation of currents in response to 10 μM GABA (I_{GABA}) in type I neurones by etifoxine. The results are expressed as mean±s.d. from six different cells and the amplitudes were normalised to the control GABA current which was recorded in the absence of etifoxine and fixed as 100%. Note the bell-shaped dependence of the potentiating effect. The numbers in the columns are the values of the mean for each condition. (c) Similar protocol as in (a) but for a neurone displaying a small current (~10 pA) in response to 10 μM GABA. These neurones were classified as type II neurones. Note a very strong potentiating effect for etifoxine concentrations >0.5 μM. (d) Summary of the potentiating effect of increasing concentrations of etifoxine on GABA currents induced by 10 μM GABA (I_{GABA}) in eight type II neurones. Results are expressed as mean±s.d. Since the amplitude of the control GABA current was very small, the data were normalised with respect to the GABA response recorded in the presence of 2 μM etifoxine which was set to 100%. Note that in type II neurones the bell-shaped dose–response relationship for etifoxine was less pronounced. In all cases, the holding potential was −40 mV.

3.4. Etifoxine modulates an endogenous GABAergic tone

3.4.1. Existence of a GABAergic tone

In all cells in which it was tested (*n*=9), bath application of the competitive $GABA_A$ receptor antagonist bicuculline (10 μM), reversibly reduced the amplitude of the holding current necessary to maintain the membrane potential at −60 mV, a phenomenon which was accompanied by a marked reduction in membrane noise (Fig. 2a). When spontaneous or miniature IPSCs were present, they were also reversibly blocked by bicuculline indicating that they were mediated by the activation of $GABA_A$ receptors (Fig. 2a). These results indicated that under resting conditions, and despite the continuous perfusion of the preparation with extracellular solution, there existed a constant $GABA_A$-receptor-mediated tone (current) probably due to the presence of a low concentration of GABA (or alternatively of a GABA-like substance) in the extracellular space. The mean amplitude of this bicuculline-sensitive current was −17.2±8.1 pA at a holding potential of −60 mV (*n*=9).

3.5. Induction of an inward current by etifoxine

Application of etifoxine (30 μM) induced a slowly developing inward current which was systematically

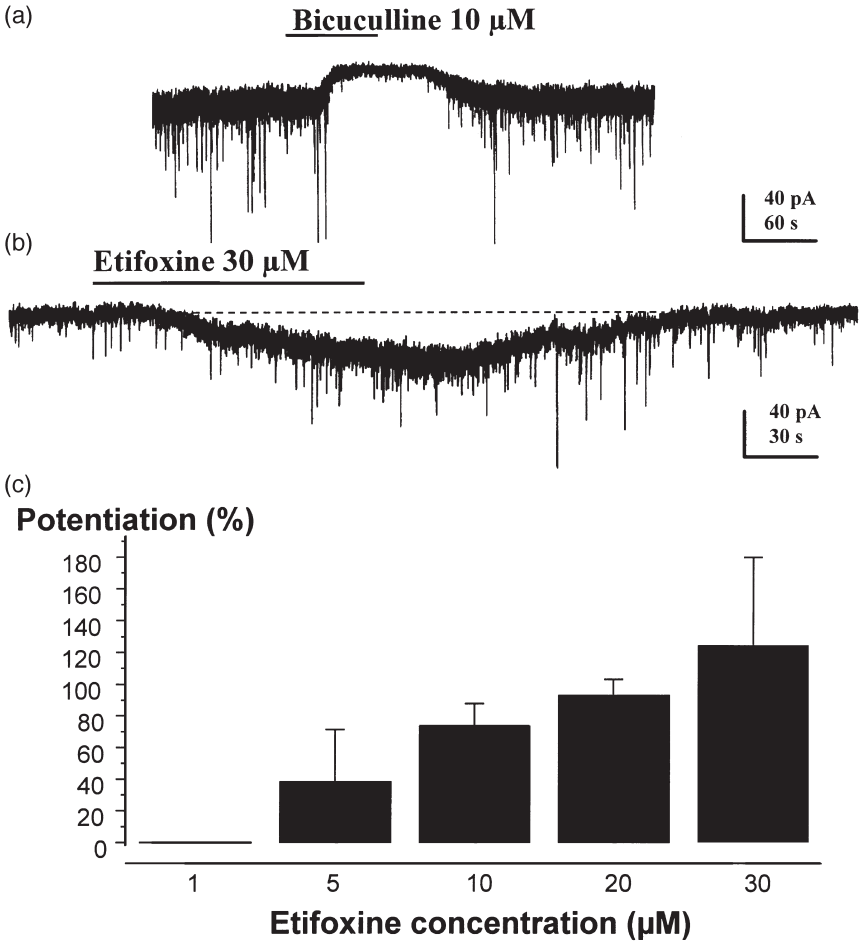

Fig. 2. Etifoxine modulates a tonic $GABA_AR$-mediated current in cultured hypothalamic neurones. (a) Application of bicuculline (10 μM) reversibly reduced the current necessary to hold the membrane potential of the cell at −60 mV. This phenomenon was accompanied by a marked reduction of the membrane noise indicating that bicuculline was closing $GABA_AR$ channels open under resting conditions and responsible for a constant post-synaptic GABAergic tone. Bicuculline also reversibly blocked the synaptic currents (fast downward deflections of the current trace) recorded in hypothalamic neurones indicating that these were $GABA_AR$-mediated inhibitory post-synaptic currents (IPSCs). (b) Etifoxine (30 μM), applied for the duration indicated by the horizontal bar, induced a slowly developing inward current which was accompanied by an increase in membrane noise and of IPSC frequency. This current reached a maximum during the early washout period and returned progressively to baseline (stippled horizontal line). (c) Percentage of potentiation of the bicuculline-sensitive component of membrane current by increasing concentrations of etifoxine. The amplitude of the bicuculline-sensitive component was determined as the amplitude of the current suppressed by bicuculline (see panel a). After having washed out bicuculline, etifoxine was applied and the amplitude of the current induced by etifoxine was normalised with respect to the amplitude of the bicuculline-sensitive current (which was set to 100%). Note that etifoxine potentiated this tonic GABAergic current in a dose-dependent manner for concentrations >1 μM. The holding potential was −60 mV.

accompanied by an increase in membrane noise (Fig. 2b) in 85% of the neurones tested (n=32). When spontaneous or miniature IPSCs were recorded, their frequency was increased in the majority of cases (70%, n=20). An inward current induced/modulated by etifoxine was also occasionally recorded in neurones receiving no functional synaptic input (n=4), as attested by the absence of IPSCs even after the local application of a 'depolarising' extracellular solution containing 50 mM K^+ ions which normally stimulates the Ca^{2+}-dependent release of GABA in the same system (Poisbeau et al., 1996). As shown in Fig. 2c, the amplitude of the current increased with the concentration of etifoxine. At a holding potential of −60 mV, the mean amplitude of the current induced by 30 μM etifoxine was −43.1±13.7 pA (n=11). The current appeared with a mean delay of 1.3±0.3 min (n=5) after onset of bath application and reached its maximal amplitude during the early period of rinsing before returning to baseline on average after 4.6±1.6 min (n=5). Such a current was never observed in the presence of bicuculline (n=9).

3.6. Effect of antagonists of central and peripheral benzodiazepine receptors

Flumazenil (10 μM), an antagonist of central-type benzodiazepine sites at $GABA_A$ receptors (Sigel and Buhr, 1997), did not affect the amplitude of the current

induced by 30 μM etifoxine (n=7). The mean amplitudes of the etifoxine current were −41.6±5.2 pA (n=7) under control conditions and −41±6.6 pA (n=7) in the presence of flumazenil (10 μM). These values were not statistically different (T-test, $P>0.05$).

We next tested the effect of PK11195, an antagonist of peripheral (mitochondrial)-type benzodiazepine receptors (Benavides et al., 1983; Mensah-Nyagan et al., 1999). These receptors, which are highly expressed in glial cells, are not associated with $GABA_A$ receptors but promote the transport of cholesterol through the inner membrane of mitochondria thereby stimulating the synthesis of neurosteroids which are potent allosteric modulators at $GABA_A$ receptors (Papadopoulos, 1993; Robel and Beaulieu, 1994; Lambert et al., 1995; Mensah-Nyagan et al., 1999). PK11195 applied alone at a maximal concentration of 10 μM did not induce any current (n=4) indicating that (i) it did not directly interfere with $GABA_A$ receptors and (ii) it did not modify the basal GABAergic tone (see preceding section). Moreover, PK11195 (10 μM) did not modify the amplitude of the current induced by local application (by means of the U-tube) of exogenous GABA (5 μM, n=5). However, at the same concentration (10 μM), PK11195 reversibly inhibited the amplitude of the membrane current induced by 30 μM etifoxine (Fig. 3a). This effect was observed in the presence of flumazenil (10 μM) and was similar during either acute application of PK11195 or preincubation of the cells with PK11195 (Fig. 3b). For acute application experiments, the amplitudes of the etifoxine (30 μM) currents were −41±13.3 pA and −15.4±10.8 pA (n=4) in the absence and in the presence of PK11195 (10 μM) respectively. These values were significantly different ($P<0.05$). Similarly, for preincubation experiments, the amplitudes of the currents induced by etifoxine (30 μM) were −41±6.6 pA (n=5) in the absence of PK11195 and −16.6±3.1 pA (n=5) when the cultures were preincubated with PK11195 (10 μM) for at least 1 hr before recording. Thus, PK11195 (10 μM) blocked about 60% of the current induced by 30 μM etifoxine. Taken together, our results suggest that etifoxine exerts a direct positive allosteric modulation at $GABA_A$ receptors. In addition, it seems that at least part of the positive modulatory action of etifoxine on the slow $GABA_A$-receptor-mediated current was due to an indirect mechanism involving the activation of PK11195-sensitive peripheral benzodiazepine receptors.

3.7. Effect of etifoxine on GABAergic IPSCs

In 63% (10 out of 16) of the neurones in which etifoxine (10–30 μM) induced a post-synaptic inward current we also observed an increase in the frequency of GABAergic IPSCs. In some rare cases (11%, 2 out of 18 neurones), a stimulation of the IPSC frequency was observed in the absence of any detectable post-synaptic etifoxine-induced current. The mean increase in frequency was of 94±31% (n=10) for spontaneously occurring IPSCs (sIPSCs) and of 187.8±106.8% (n=4) for miniature IPSCs (mIPSCs), the latter being recorded in the presence of 0.5 μM tetrodotoxin (TTX, Fig. 4a and b). The mean increases in frequency in both situations were not significantly different ($P>0.05$). When the results on sIPSCs and mIPSCs were pooled, the mean increase in frequency was 120.8±79.3% (n=14). The slowly developing and recovering inward current evoked by etifoxine (30 μM) was still detected in the presence of TTX Fig. 4a and its amplitude (−30±21.8 pA, n=3) was comparable to and not significantly different from ($P>0.05$) that recorded in the absence of TTX (−43.1±13.7 pA, n=11). The kinetics of the decay phases of the majority (70%) of sIPSCs and mIPSCs could be satisfactorily described by a single exponential function (Fig. 4c), with a time constant of τ=36±8.4 ms (n=10) for sIPSCs and of τ=35.7±5.7 ms (n=4) for mIPSCs, these values being not significantly different ($P>0.05$). In a subset (30%) of sIPSCs and mIPSCs two exponential functions were required to describe their decay phases (Fig. 4c). The time constants τ_f (fast component) and τ_s (slow component) of these functions were: τ_f=3.3±1.3 ms and τ_s=47.3±8.0 ms (n=10) for sIPSCs and τ_f=3.0±1.7 ms and τ_s=50.0±9.2 ms (n=4) for mIPSCs (Fig. 4c). In the presence of etifoxine (30 μM), these time constants were τ_f=3.3±1.3 ms and τ_s=44.2±9.6 ms (n=10) for sIPSCs and τ_f=2.3±0.4 ms and τ_s=42.6±4.5 ms (n=4) for mIPSCs. The values of the time constants of sIPSCs and mIPSCs were not significantly different from those of sIPSCs and mIPSCs recorded under control conditions, i.e in the absence of etifoxine ($P>0.05$). Moreover, etifoxine did not affect the amplitude distribution or the decay time constants of sIPSCs or mIPSCs (Fig. 4d). The mean amplitudes of sIPSCs and mIPSCs were −48.0±22.7 pA (n=10) and −31.9±22.3 pA (n=4) respectively under control conditions and −48.0±18.0 pA (n=10) and −32.5±21.2 pA (n=4) in the presence of 30 μM etifoxine.

After preincubation of the cells with PK11195 (10 μM) for at least 1 hr, the percentage of neurones responding to etifoxine by an increase in IPSC frequency was lower (37.5%, n=8) than under control conditions (75%, n=16) although all cells tested in the presence of PK1115 displayed IPSCs indicating that they received a functional GABAergic synaptic input. In addition, in the three cells in which an increase in IPSC frequency was detected in the presence of PK11195, the mean percentage of increase (29.3±3.2%) was significantly smaller ($P<0.05$) than that observed under control conditions, i.e. in standard extracellular solution or in the presence of TTX (see above).

These effects of etifoxine were similar to those of the neurosteroid allopregnanolone, previously described in the same preparation (Poisbeau et al., 1997), and are

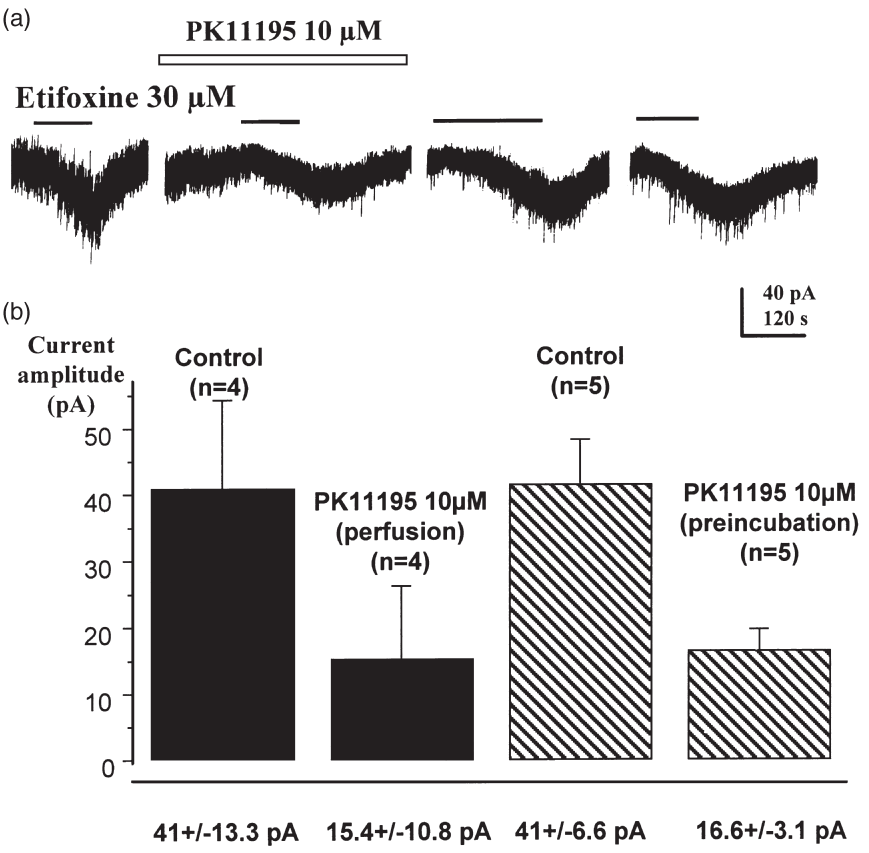

Fig. 3. Effect of the peripheral (mitochondrial)-type benzodiazepine receptor antagonist PK11195 on the membrane current induced by 30 μM etifoxine. (a) Application of etifoxine (30 μM) for the duration indicated by the horizontal black bar induced a slow and reversible inward current which was accompanied by an increase in membrane noise. After switching to an extracellular medium containing 10 μM PK11195 (horizontal white bar), the current induced by etifoxine was inhibited by about 60%. This inhibition was reversible after washing out PK11195 from the extracellular medium. (b) Comparison of the inhibitory effects of PK11195 on the etifoxine-induced current after either acute perfusion (black columns) or after preincubation of the cells for at least 1 hr with PK11195 (10 μM) before testing etifoxine (hatched columns). For acute perfusion, the amplitudes of the control currents could be compared directly with those in the presence of PK11195 in the same cells. The mean inhibition was 62% and the two means were significantly different ($P<0.05$). Similar observations were made for preincubation experiments, although the control current and that measured in the presence of PK11195 were not determined in the same cells. The mean inhibition was 60% and the two means were significantly different ($P<0.05$). The numbers below the columns are the mean values±s.d. of the amplitudes of the etifoxine-induced currents. All experiments were performed in the presence of flumazenil (10 μM) in the extracellular medium and the holding potential was −60 mV.

consistent with a facilitatory presynaptic action of etifoxine on GABA release.

4. Discussion

The results of our study indicate that etifoxine potentiates $GABA_AR$ function and facilitates GABAergic synaptic transmission in cultured hypothalamic neurones. Etifoxine appeared to have a dual mode of action on these neurones which included (i) a direct positive allosteric modulation of $GABA_ARs$ through a site distinct from that of benzodiazepines and (ii) an indirect effect which might involve the stimulation of peripheral (mitochondrial)-type benzodiazepine receptors known to control neurosteroid synthesis (Lambert et al., 1995; Mensah-Nyagan et al., 1999). Pre- and/or post-synaptic potentiating actions of etifoxine on GABAergic synaptic transmission similar to those reported here on hypothalamic neurones might account for the anxiolytic effect of etifoxine observed in behavioural tests.

4.1. Etifoxine potentiates $GABA_AR$-mediated currents

Etifoxine potentiated the membrane currents induced by submaximal concentrations of GABA but did not modify the response to saturating agonist concentrations. This phenomenon was observed both for isolated DH neurones and for cultured hypothalamic neurones and was consistent with an increase in affinity of $GABA_ARs$ as observed in various types of neurones with other positive allosteric modulators of $GABA_ARs$ such as benzodiazepines, barbiturates or neurosteroids (Macdonald and Olsen, 1994; Sieghart, 1995; Mehta and Ticku, 1999). Benzodiazepine sites present at $GABA_ARs$ were certainly not involved in this allosteric modulation since the

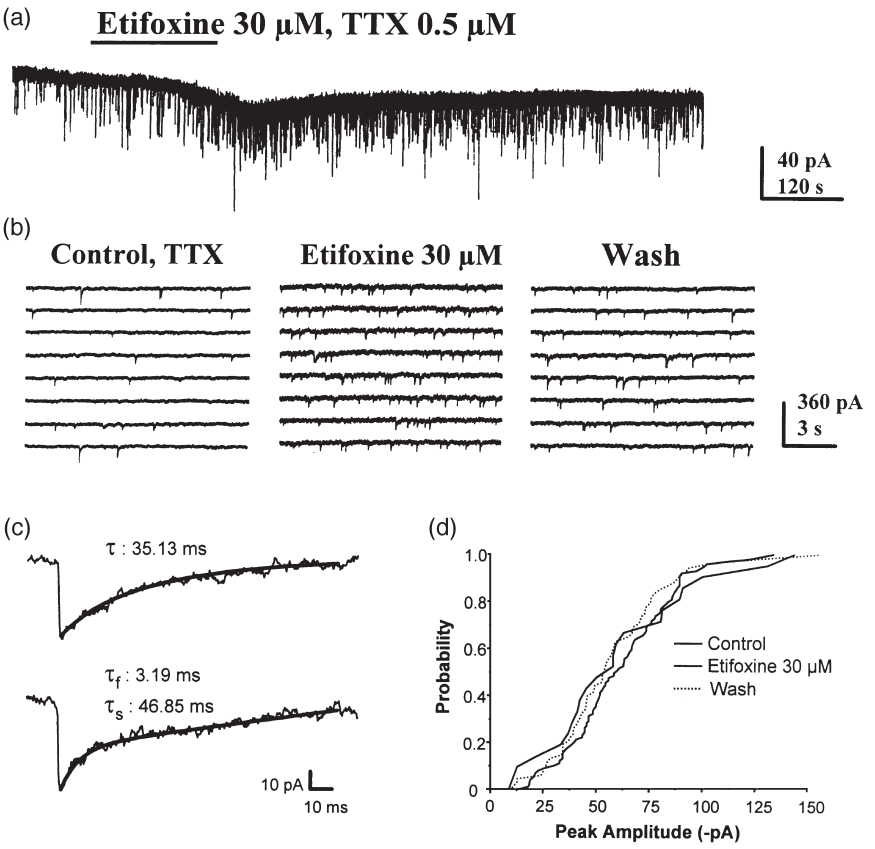

Fig. 4. Effect of etifoxine (30 μM) on miniature GABAergic inhibitory post-synaptic currents (mIPSCs) recorded in the presence of 0.5 μM tetrodotoxin (TTX). (a) In the presence of TTX, application of etifoxine (30 μM) for the duration indicated by the horizontal bar elicited an inward current which was accompanied by an increase in membrane noise and an augmentation in the frequency of mIPSCs (fast downward deflections of the current trace). (b) Current traces represented on an expanded time scale and corresponding each to a sequence of 90 sec of mIPSCs under control conditions (left panel), in the presence of 30 μM etifoxine (middle panel) and after washout of the substance (right panel). Note the pronounced and reversible increases in membrane noise and of the mIPSC frequency in the presence of etifoxine. (c) Kinetic properties of mIPSCs. The majority (70%) of mIPSCs decay phases could be fitted with a single exponential function (top trace) whereas about 30% of the synaptic events required two exponential functions (bottom trace). The values of the time constants τ are indicated. τ_s and τ_f correspond respectively to the fast and slow components of biexponential mIPSCs. The thick lines superimposed on the mIPSC traces are the results of the fits. (d) Cumulative probability histogram of mIPSC amplitude distribution in the absence (control, wash) and in the presence of etifoxine (30 μM). Note that etifoxine did not affect the overall distribution of mIPSC amplitudes. All data are from the same cell. The holding potential was −60 mV.

effect of etifoxine was still observed in the presence of 10 μM flumazenil (data not shown), a selective antagonist of central-type benzodiazepine sites.

The concentration-clamp technique allowed fast application of pharmacological substances to single DH neurones, i.e. physically isolated from surrounding glial cells or neurones. Under these conditions, the observed modulatory effects of etifoxine could only be attributed to a direct action on the recorded neurone. At concentrations up to 30 μM, etifoxine did not induce any membrane current even when applied for 1–2 min, suggesting that it was probably not a $GABA_AR$ agonist. Unfortunately, higher concentrations of etifoxine could not be tested because of the low solubility of this substance in physiological extracellular solution at concentrations >30 μM. We therefore cannot completely exclude the possibility that etifoxine might act as a partial agonist at high concentrations, although such concentration are not likely to be reached in the cerebrospinal fluid due to the low solubility of etifoxine. The threshold concentration of etifoxine for potentiating $GABA_AR$ currents was around 0.1 μM, which was consistent with our binding data indicating that etifoxine displaced [^{35}S]TBPS from $GABA_ARs$ with an IC_{50} of 7 μM. From a functional point of view, we noted a maximal potentiating effect for 2 μM etifoxine. At higher concentrations the potentiating effect was either decreased (type I DH neurones, Fig. 1a and b) or maintained at a plateau level (type II DH neurones, Fig. 1c and d). It must be emphasised that in type I neurones the amplitude of $GABA_AR$ currents was never decreased below that of control currents recorded in the absence of etifoxine. Although we have no clear explanation for the difference in effect of etifoxine in type I and type II neurones, it was a consistent observation that the amplitudes of control currents in response to 10 μM GABA were much larger in type I neurones (~365 pA)

than in type II neurones (~10 pA). There was no obvious difference in current desensitisation in the presence of etifoxine between the two types of neurones. Although it cannot be excluded that the two types of neurones differ by the subunit composition of their $GABA_A$ receptors, it must be emphasised that DH neurones appear to express a limited set of $GABA_AR$ subunits, i.e α_2, α_3, $\beta_{2,3}$ and γ_2 (Wisden et al., 1991; Bohlhalter et al., 1996). The functional properties of these receptors are therefore likely to be relatively similar. One possible explanation that could account for our observations is a different degree of phosphorylation of $GABA_ARs$ receptors in type I and II neurones. Indeed, phosphorylation is known to modulate the activity of $GABA_ARs$ (Macdonald and Olsen, 1994) and this point will have to be addressed in more detail in future studies.

4.2. Etifoxine modulates a GABAergic tone in hypothalamic neurones and facilitates GABAergic synaptic transmission

In 85% of the hypothalamic neurones, the potentiating effect of submaximal GABA currents was accompanied by a slowly developing, dose-dependent, inward current during the superfusion of etifoxine. This current was probably not due to the direct (agonist-like) activation of $GABA_ARs$ since local application of etifoxine (30 μM) by means of the U-tube did not induce a fast inward current. Moreover, the etifoxine-induced current was slow in onset and persisted in most cases for several minutes after washout of the substance. Such a slow reversal would not be expected for a low-affinity (fast-dissociating) partial agonist at $GABA_A$ receptors. Finally, it must be noted that bath-applied etifoxine did not induce an inward current in all neurones tested despite all of them possessing functional $GABA_ARs$.

One major difference was that hypothalamic neurones, unlike DH neurones, were not physically isolated from neighbouring glial cells during recordings. We noticed that in all neurones tested, the application of bicuculline (10 μM) alone, i.e in the absence of etifoxine or GABA, reduced the current necessary to hold the cell at −60 mV as well as the membrane noise, indicating that bicuculline was closing membrane channels. Since bicuculline is a competitive antagonist at $GABA_ARs$, this observation suggested that there was a low level of GABA continuously present in the extracellular environment, i.e. that there was a constant GABAergic tone at rest which persisted despite continuous perfusion of the cultures with extracellular medium. Similar situations have been reported for GABA in cerebellar slices (Kaneda et al., 1995) and hippocampal cultures (Vautrin et al., 1993). Therefore, the current apparently induced by etifoxine could in fact reflect the positive allosteric modulation of this tonic GABAergic component. Consistent with this, we never observed any etifoxine-induced current in the presence of bicuculline. The etifoxine current was not affected by flumazenil indicating that etifoxine did not act via central-type benzodiazepine sites at $GABA_ARs$. Unfortunately, specific antagonists of barbiturate or neurosteroid sites at $GABA_ARs$ are not available at present and therefore we do not know if one of these two sites (or alternatively a distinct site) might be involved in the effect of etifoxine.

4.3. Speculation on a possible link between etifoxine and neurosteroids

We noted strong similarities between the potentiation of $GABA_AR$ currents, the increase in membrane noise, the slowly developing inward current and the presynaptic facilitatory effect on GABAergic IPSCs (see below) induced by etifoxine and that induced by the neurosteroid allopregnanolone under the same experimental conditions (Poisbeau et al., 1997).

Indeed, an important step in controlling the synthesis of neurosteroids is the transport of cholesterol across the inner membrane of mitochondria (Papadopoulos, 1993; Lambert et al., 1995; Mensah-Nyagan et al., 1999). This phenomenon is stimulated by the binding of endogenous peptides such as endozepines or of some exogenous benzodiazepines (e.g. diazepam) to a receptor located on mitochondria (and possibly on plasma membrane; Oke et al., 1992; Mensah-Nyagan et al., 1999) termed peripheral (mitochondrial)-type benzodiazepine receptor (Papadopoulos, 1993). These receptors are selectively antagonized by PK11195 (Benavides et al., 1983; Mensah-Nyagan et al., 1999) and are different from central-type benzodiazepine sites located on $GABA_ARs$. In hypothalamic cultures, application of PK11195 at a concentration sufficient to block peripheral (mitochondrial)-type benzodiazepine receptors inhibited neither the basal GABAergic tone nor the membrane current induced by local exogenous application of GABA (5 μM). Moreover, PK11195 had no effect on the current evoked by 10 μM GABA in DH neurones (n=5, data not shown). However, at the same concentration, PK11195 reversibly reduced, by about 60%, the amplitude of the etifoxine-induced current in hypothalamic neurones. Interestingly, PK11195 also inhibited the presynaptic increase in IPSC frequency induced by etifoxine, suggesting the existence of a common PK11195-sensitive step in the post- and presynaptic effects of etifoxine. The effect of PK11195 was in line with our binding data showing that etifoxine displaced [^{3}H]PK11195 from peripheral (mitochondrial) benzodiazepine sites (IC_{50}=27 μM). Moreover, the effects of etifoxine were virtually identical to those of allopregnanolone in the same preparation (Poisbeau et al., 1997) which included a potentiation of submaximal but not saturating GABA responses and an increase in spontaneous and miniature IPSCs without any change in the kinetic characteristics or the amplitude distribution

of these IPSCs. It is therefore very tempting, though speculative, to suggest that etifoxine could modulate neurosteroid production by stimulating directly or indirectly peripheral benzodiazepine receptors. A direct proof of this hypothesis would require the measurement of locally produced neurosteroids, which is technically impossible at present. Alternatively, specific antagonists of the neurosteroid site at $GABA_A$ receptors would be very helpful. Unfortunately, such antagonists are not yet available.

4.4. Possible physiological and clinical significance

The results presented above suggest that the GABAergic tone due to the constant presence of a low concentration of GABA (or of a GABA-like substance) might be an interesting target for both physiological and clinical (pharmacological) modulations. As a first approximation one could suggest that any increase in the accumulation of extracellular GABA would reinforce this tonic GABAergic inhibition, whereas any decrease in extracellular GABA levels would have the opposite effect. Alternatively, one could envisage modulating the affinity of the $GABA_A$Rs which underlie this GABAergic tone without altering the concentration of GABA in the extracellular space. This could in principle be achieved with synthetic or natural allosteric modulators such as benzodiazepines or neurosteroids (Macdonald and Olsen, 1994; Sieghart, 1995; Mehta and Ticku, 1999). Neurosteroids seem to be particularly promising in this respect since, in contrast to benzodiazepines, they appear to be devoid of major side-effects (Gasior et al., 1999). Interestingly, a reduction in CNS levels of allopregnanolone has been noted in patients with major depression and the restoration of normal levels after treatment with fluoxetine was shown to correlate well with therapeutic improvement (Uzunova et al., 1998). In this context, a molecule such as etifoxine might be of interest since it combines a direct positive allosteric modulatory action on $GABA_A$Rs and possibly, a stimulatory action on peripheral (mitochondrial)-type benzodiazepine receptors which are associated with steroid/neurosteroid genesis (Papadopoulos, 1993; Mensah-Nyagan et al., 1999). Interestingly, CNS tissues also rapidly metabolise neurosteroids (Majewska, 1992; Gasior et al., 1999). Under these conditions the effect of neurosteroids on $GABA_A$Rs will depend essentially on the balance between their synthesis and degradation (transformation). This balance might be a target for synthetic molecules but also, in a physiological context, for endogenous molecules such as neurotransmitters or neuropeptides (e.g. peptidic transmitters or endozepines). Moreover, depending on the enzymatic equipment of cells, different types of neurosteroids are likely to be produced by different cells in the same tissue (Majewska, 1992; Robel and Beaulieu, 1994; Lambert et al., 1995; Mensah-Nyagan et al., 1999). Among these, some might act as positive and others as negative modulators, as has been shown for allopregnanolone and pregnenolone sulphate respectively on GABAergic synaptic transmission (Majewska, 1992; Poisbeau et al., 1997). Therefore, selective and differential modulation of pathways producing neurosteroids with opposite allosteric effects might contribute to the fine tuning of synaptic transmission in the CNS under physiological conditions and therefore represent an interesting target for clinically active molecules.

Acknowledgements

We wish to thank D. Di Scala, Y. Schwab and E. Jover for providing hypothalamic cultures, and Roche (Basel, Switzerland) for the gift of flumazenil. Many thanks to Yves De Konninck (McGill University, Montreal, Canada) for making available to us his synaptic current analysis program. This work was supported by University Louis Pasteur, CNRS (Centre National de la Recherche Scientifique), and Biocodex Laboratories.

References

Benavides, J., Quarteronet, D., Imbault, F., Malgouris, C., Uzan, A., Renault, C. et al., 1983. Labelling of "peripheral-type" benzodiazepine binding sites in the rat brain by using [^{3}H]PK11195, an isoquinoline carboxamide derivative: kinetic studies and autoradiographic localization. Journal of Neurochemistry 41, 1744–1750.

Bohlhalter, S., Weinmann, O., Mohler, H., Fritschy, J.M., 1996. Laminar compartmentalization of $GABA_A$-receptor subtypes in the spinal cord: an immunohistochemical study. Journal of Neuroscience 16, 283–297.

Boissier, J.R., Simon, P., Zaczinska, M., Fichelle, J., 1972. Etude psychopharmacologique expériementale d'une nouvelle substance psychotrope, la éthylamino-6-chloro-4-méthyl-4-phényl-4*H*-3,1-benzoxazine. Thérapie 27, 325–338.

Bottenstein, J.E., Sato, G.H., 1979. Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proceedings of the National Academy of Sciences of the United States of America 76, 514–517.

Davies, P.A., Hanna, M.C., Hales, T.G., Kirkness, E.F., 1997. Insensitivity to anaesthetic agents conferred by a class of GABA(A) receptor subunits. Nature 385, 820–823.

Defazio, T., Hablitz, J.J., 1998. Zinc and zolpidem modulate mIPSCs in rat neocortical pyramidal neurons. Journal of Neurophysiology 80, 1670–1677.

Fenwick, E.M., Marty, A., Neher, E., 1982. A patch-clamp study of bovine chromaffin cells and of their sensitivity to acetylcholine. Journal of Physiology (London) 331, 577–597.

Gasior, M., Carter, R.B., Witkin, J.M., 1999. Neuroactive steroids: potential therapeutic use in neurological and psychiatric disorders. Trends in Pharmacological Sciences 20, 107–112.

Hall, M.D., El Mestikawy, S., Emerit, M.B., Pichat, L., Hamon, M., Gozlan, H., 1985. [^{3}H]8-Hydroxy-2-(di-*n*-propylamino)tetralin binding to pre- and postsynaptic 5-hydroxytryptamine sites in various regions of the rat brain. Journal of Neurochemistry 44, 1685–1696.

Harrison, N.L., Vicini, S., Barker, J.L., 1987. A steroid anesthetic prolongs inhibitory postsynaptic currents in cultured rat hippocampal neurons. Journal of Neuroscience 7, 604–609.

Kaneda, M., Farrant, M., Cull-Candy, S.G., 1995. Whole-cell and single-channel currents activated by GABA and glycine in granule cells of the rat cerebellum. Journal of Physiology (London) 485, 419–435.

Kruse, H.J., Kuch, H., 1985. Etifoxine: evaluation of its anticonvulsant profile in mice in comparison with sodium valproate, phenytoin and clobazam. Arzneimittelforschung 35, 133–135.

Lambert, J.J., Belelli, D., Hill-Venning, C., Peters, J.A., 1995. Neurosteroids and $GABA_A$ receptor function. Trends in Pharmacological Sciences 16, 295–303.

Le Fur, G., Vaucher, N., Perrier, M.L., Flamier, A., Benavides, J., Renault, C. et al., 1983. Differentiation between two ligands for peripheral benzodiazepine binding sites, [^{3}H]RO5-4864 and [^{3}H]PK 11195, by thermodynamic studies. Life Sciences 33, 449–457.

Lewin, A.H., de Costa, B.R., Rice, K.C., Skolnick, P., 1989. *meta*- and *para*-Isothiocyanato-*t*-butylbicycloorthobenzoate: irreversible ligands of the gamma-aminobutyric acid-regulated chloride ionophore. Molecular Pharmacology 35, 189–194.

Leysen, J.E., Niemegeers, C.J., Van Nueten, J.M., Laduron, P.M., 1982. [^{3}H]Ketanserin (R 41 468), a selective ^{3}H-ligand for serotonin2 receptor binding sites. Binding properties, brain distribution, and functional role. Molecular Pharmacology 21, 301–314.

Macdonald, R.L., Olsen, R.W., 1994. $GABA_A$ receptor channels. Annual Reviews in Neuroscience 17, 569–602.

Majewska, M.D., 1992. Neurosteroids: endogenous bimodal modulators of the $GABA_A$ receptor. Mechanism of action and physiological significance. Progress in Neurobiology 38, 379–395.

Malcangio, M., Bowery, N.G., 1996. GABA and its receptors in the spinal cord. Trends in Pharmacological Sciences 17, 457–462.

Mehta, A.K., Ticku, M.K., 1999. An update on $GABA_A$ receptors. Brain Research: Brain Research Reviews 29, 196–217.

Mensah-Nyagan, A.G., Do-Rego, J.L., Beaujean, D., Luu-The, V., Pelletier, G., Vaudry, H., 1999. Neurosteroids: expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. Pharmacological Reviews 51, 63–81.

Oke, B.O., Suarez-Quian, C.A., Riond, J., Ferrara, P., Papadopoulos, V., 1992. Cell surface localization of the peripheral-type benzodiazepine receptor (PBR) in adrenal cortex. Molecular and Cellular Endocrinology 87, R1–6.

Papadopoulos, V., 1993. Peripheral-type benzodiazepine/diazepam binding inhibitor receptor: biological role in steroidogenic cell function. Endocrinology Reviews 14, 222–240.

Perrais, D., Ropert, N., 1999. Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic $GABA_A$ receptors in central synapses. Journal of Neuroscience 19, 578–588.

Poisbeau, P., Feltz, P., Schlichter, R., 1997. Modulation of $GABA_A$ receptor-mediated IPSCs by neuroactive steroids in a rat hypothalamo-hypophyseal coculture model. Journal of Physiology (London) 500 (Part 2), 475–485.

Poisbeau, P., Rene, F., Egles, C., Felix, J., Feltz, P., Schlichter, R., 1996. Characterization of functional GABAergic synapses formed between rat hypothalamic neurons and pituitary intermediate lobe cells in coculture: Ca^{2+} dependence of spontaneous IPSCs. Journal of Neuroscience 16, 4835–4845.

Pritchett, D.B., Sontheimer, H., Shivers, B.D., Ymer, S., Kettenmann, H., Schofield, P.R. et al., 1989. Importance of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature 338, 582–585.

Robel, P., Beaulieu, E.E., 1994. Neurosteroids: biosynthesis and function. Trends in Endocrinology and Metabolism 5, 1–7.

Rybalchenko, V., Schlichter, R., 1999. Functional high- and low-affinity agonist binding sites at native dorsal horn AMPA receptors. Neuroreport 10, 1803–1806.

Servant, D., Graziani, P.L., Moyse, D., Parquet, P.J., 1998. Traitement du trouble de l'adaptation avec anxiété: évaluation de l'efficacité et de la tolérance de l'étifoxine par un essai en double aveugle contre produit de référence. L'Encéphale 24, 569–574.

Shank, R.P., Baldy, W.J., Mattucci, L.C., Villani, F.J. Jr., 1990. Ion and temperature effects on the binding of gamma-aminobutyrate to its receptors and the high-affinity transport system. Journal of Neurochemistry 54, 2007–2015.

Sieghart, W., 1995. Structure and pharmacology of gamma-aminobutyric acid A receptor subtypes. Pharmacological Reviews 47, 181–234.

Sigel, E., Buhr, A., 1997. The benzodiazepine binding site of $GABA_A$ receptors. Trends in Pharmacological Sciences 18, 425–429.

Snodgrass, S.R., 1978. Use of ^{3}H-muscimol for GABA receptor studies. Nature 273, 392–394.

Speth, R.C., Wastek, G.J., Yamamura, H.I., 1979. Benzodiazepine receptors: temperature dependence of [^{3}H]flunitrazepam binding. Life Sciences 24, 351–357.

Tejani-Butt, S.M., 1992. [^{3}H]Nisoxetine: a radioligand for quantitation of norepinephrine uptake sites by autoradiography or by homogenate binding. Journal of Pharmacology and Experimental Therapeutics 260, 427–436.

Uzunova, V., Sheline, Y., Davis, J.M., Rasmusson, A., Uzunov, D.P., Costa, E. et al., 1998. Increase in the cerebrospinal fluid content of neurosteroids in patients with unipolar major depression who are receiving fluoxetine or fluvoxamine. Proceedings of the National Academy of Sciences of the United States of America 95, 3239–3244.

Vautrin, J., Schaffner, A.E., Barker, J.L., 1993. Tonic GABA secretion of cultured rat hippocampal neurons rapidly transformed by Zn^{2+} into quantal release. Neuroscience Letters 158, 125–129.

Vogel, J.R., Beer, B., Clody, D.E., 1971. A simple and reliable conflict procedure for testing anti-anxiety agents. Psychopharmacologia 21, 1–7.

Wisden, W., Gundlach, A.L., Barnard, E.A., Seeburg, P.H., Hunt, S.P., 1991. Distribution of $GABA_A$ receptor subunit mRNAs in rat lumbar spinal cord. Brain Research: Molecular Brain Research 10, 179–183.

Zhu, W., Wang, J., Krueger, K., Vicini, S., 1996. Delta subunit inhibits neurosteroid modulation of $GABA_A$ receptors. Journal of Neuroscience 16, 6648–6656.