Research Report

Neurosteroid allopregnanolone mediates anxiolytic effect of etifoxine in rats

Rajesh R. Ugale\textsuperscript{b,c}, Ajaykumar N. Sharma\textsuperscript{a,b}, Dadasaheb M. Kokare\textsuperscript{a}, Khemraj Hirani\textsuperscript{a,d}, Nishikant K. Subhedar\textsuperscript{a}, Chandrabhan T. Chopde\textsuperscript{a,b,*}

\textsuperscript{a}Department of Pharmaceutical Sciences, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur-440 033, Maharashtra, India
\textsuperscript{b}Pharmacology Division, Shrimati Kishoritai Bhoyar College of Pharmacy, New Kamptee-441 002, Nagpur, Maharashtra, India
\textsuperscript{c}Department of Psychiatry, University of Illinois at Chicago, Chicago, Illinois 60612, USA
\textsuperscript{d}Department of Anatomy, College of Medicine, University of South Florida, Health Science Centre, Tampa, FL 33612-4799, USA

\textit{ARTICLE INFO}

Article history:
Accepted 17 September 2007
Available online 25 September 2007

Keywords:
Etifoxine
Neurosteroids
Allopregnanolone
Anxiety

\textit{ABSTRACT}

Etifoxine (6-chloro-2-ethylamino-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride), a nonbenzodiazepine anxiolytic drug, potentiates GABA\textsubscript{A} receptor function perhaps through stimulation of neurosteroid biosynthesis. However, the exact mechanism of etifoxine action is not fully understood. In this study, we have assessed the possible role of GABA\textsubscript{A}ergic neurosteroid like allopregnanolone (ALLO) in the anxiolytic-like effect of etifoxine in rats using elevated plus maze test. Selective GABA\textsubscript{A} receptor agonist, muscimol, ALLO or neurosteroidogenic agents like progesterone, metyrapone or mitochondrial diazepam binding inhibitor receptor (MDR) agonist, FGIN 1-27 significantly heightened the etifoxine-induced anxiolysis. On the other hand, GABA\textsubscript{A} receptor antagonist, bicuculline or neurosteroid biosynthesis inhibitors like finasteride, indomethacin, trilostane or PBR antagonist, PK11195 significantly blocked the effect of etifoxine. Bilateral adrenalectomy did not influence anti-anxiety effect of etifoxine thereby ruling out contribution of adrenal steroids. Thus, our results provide behavioral evidence for the role of neurosteroids like ALLO in the anti-anxiety effect of etifoxine.

© 2007 Elsevier B.V. All rights reserved.

\textit{Keywords:} Etifoxine, Neurosteroids, Allopregnanolone, Anxiety

1. Introduction

Etifoxine (6-chloro-2-ethylamino-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride) is a nonbenzodiazepine anxiolytic in rodents (Boissier et al., 1972; Schlichter et al., 2000; Micalef et al., 2001) and in humans is effective for treating adjustment disorders with anxiety (Nguyen et al., 2006). At anxiolytic concentration, unlike classical benzodiazepines, the compound etifoxine has fewer side effects (Micalef et al., 2001; Nguyen et al., 2006). Anxiety and related disorders are considered to be associated with dysfunction of central \gammavoaminobutyric acid (GABA)ergic system (Almeida et al., 2006). Major anxiolytic drugs including benzodiazepines, barbiturates (Drafts and Fisher, 2006; Campo-Soria et al., 2006) and neurosteroids (Bitran et al., 1991, 1993; Finn et al., 1997; Akwa et al., 1999; Palmer et al., 2002) potently and selectively facilitate the action of GABA at GABA\textsubscript{A} receptors. Interestingly, etifoxine also increases GABA\textsubscript{A}ergic transmission (Schlichter et al., 2000) by acting on GABA\textsubscript{A} receptors \(\beta_2\) or \(\beta_3\) subunit (Hamon et al., 2003), an allosteric site distinct from that of...
benzodiazepines and neurosteroids (Verleye et al., 1999, 2001, 2002; Schlichter et al., 2000). In addition, etifoxine stimulates peripheral (mitochondrial)-type benzodiazepine receptors that control synthesis of neurosteroids like allopregnanolone (3α, 5α-Tetrahydroprogesterone, ALLO), an allosteric positive modulator of GABA<sub>A</sub> receptors (Harrison and Simmonds, 1984; Verleye et al., 2005).

Neurosteroids are synthesized de novo in the brain independent of peripheral steroidogenic endocrine glands (Corpechot et al., 1981). The best-characterized neurosteroids are ALLO, dehydroepiandrosterone (DHEA), pregnanolone and their sulfates etc. They interact with ionotropic receptors and ligand-gated ion channels especially GABA<sub>A</sub> receptors at nanomolar to micromolar concentration and rapidly alter the neuronal excitability (Paul and Purdy, 1992; Lambert et al., 1995; Follesa et al., 2004; Birzniece et al., 2006). ALLO is the most potent endogenous neurosteroid and is implicated in premenstrual dysphoric disorder (Klatzkin et al., 2006; N-Wihlbek et al., 2006), generalized anxiety disorder (Semeniuk et al., 2001; Hirani et al., 2005; Sharma et al., 2007), post-traumatic stress disorder (Rasmusson et al., 2006), estrous cycle (Petralia et al., 2005), pregnancy (Paoletti et al., 2006), aggression (Pinna et al., 2005) and seizures (Ugale et al., 2004a). Both ALLO and etifoxine share many features namely anti-anxiety (Boissier et al., 1972; Bitran et al., 1991, 1993; Schlichter et al., 2000; Micallef et al., 2001; Verleye et al., 2005; Nguyen et al., 2006), anti-seizure properties (Verleye et al., 2001), potentiation of GABA stimulated Cl<sup>-</sup> currents (Harrison et al., 1987; Lambert et al., 1995; Schlichter et al., 2000) or inhibition of [<sup>35</sup>S]-butylbicyclophosphorothionate ([<sup>35</sup>S]TBPS) binding (Lambert et al., 2001). The concept that etifoxine acts by stimulating neurosteroid synthesis is based on the observation that, PK11195, a compound that inhibits neurosteroid biosynthesis, partly suppresses the effect of etifoxine on GABAergic transmission or GABA<sub>A</sub> receptors (Schlichter et al., 2000; Verleye et al., 2005).

Based on earlier finding that etifoxine increases brain neurosteroidal level including ALLO (Verleye et al., 2005), we explored the role of ALLO in the anxiolytic effect of etifoxine by pharmacological manipulation of neurosteroids using elevated plus maze (EPM) test in Sprague–Dawley rats. We have recently demonstrated that several centrally active drugs produce their effects by increasing neurosteroid ALLO content in brain (Hirani et al., 2002, 2005; Ugale et al., 2004a,b; Sharma et al., 2007). Hence in the present study, we investigated the anxiolytic-like effect of etifoxine by altering the endogenous neurosteroids using neurosteroidogenic agents or its biosynthesis blockers.

2. Results

2.1. Anxiolytic effects of etifoxine and ALLO

As shown in Fig. 1, etifoxine (50 and 75 mg/kg, i.p.) or ALLO (0.5 and 1 μg/rat, i.c.v.) significantly increased the preference for open arms in the plus maze test. Etifoxine at doses 50 and 75 mg/kg increased entries [F(3,29)=42.38; P<0.0001] by 38.65% and 54.78% and time spent [F(3,29)=76.01; P<0.0001] by 47.84% and 80.01% respectively into the open arms. Similarly, ALLO (0.5 and 1 μg/rat, i.c.v.) significantly increased entries [F(3,29)=38.65; P<0.0001] into the open arms by 54.78% and 80.01% and time spent [F(3,29)=85.45; P<0.0001] in the open arms by 55.84% and 90.03% respectively. The effect of lower dose of etifoxine (25 mg/kg, i.p.) or ALLO (0.1 μg/rat, i.c.v.) 30 min prior to EPM test or aCSF (5 μl/rat, i.c.v.) 15 min prior to EPM test. Each bar represents mean±S.E.M. of data from 7 to 9 rats per group. *P<0.05 vs. vehicle control (one-way ANOVA followed by Dunnett’s test).

Fig. 1 – Dose-dependent effects of etifoxine and ALLO on (A) % open arms entries, (B) % time spent in open arms and (C) number of closed arms entries in EPM test in rats. Rats were injected etifoxine (25–75 mg/kg, i.p.) or vehicle (5 ml/kg, i.p.) 30 min prior to EPM test or ALLO (0.1–1 μg/rat, i.c.v.) or aCSF (5 μl/rat, i.c.v.) 15 min prior to EPM test. Each bar represents mean±S.E.M. of data from 7 to 9 rats per group. *P<0.05 vs. vehicle control (one-way ANOVA followed by Dunnett’s test).

2.2. ALLO or neurosteroidogenic agents potentiated the anxiolytic effect of etifoxine

Administration of subeffective dose of etifoxine (25 mg/kg, i.p.) to animals pretreated with subeffective dose of ALLO (0.1 μg/
rat, i.c.v.) showed significantly greater effect on entries \(F(3,31) = 29.335; P<0.0001\) and time spent in open arms \(F(3,31) = 41.221; P<0.0001\) (Fig. 2). The open arms entries were increased by 38.27% and the time spent in the open arms was increased by 78.18%. These treatments did not change the closed arms entries \(P>0.05\) (Fig. 2). However, the etifoxine (25 mg/kg, i.p.) and ALLO (0.1 μg/rat, i.c.v.) per se did not alter the open arm activity \(P>0.05\).

Similarly, metyrapone (50 mg/rat, i.p.), progesterone (5 mg/kg, i.p.) or FGIN 1–27 (0.5 μg/rat, i.c.v.) at the doses used here per se did not exert any significant effect on open arm parameters as compared to vehicle control groups (Fig. 2). However, at the given doses, all three drugs significantly potentiated the action of a subeffective dose of etifoxine (25 mg/kg, i.p.) on the preference for open arms activities in the plus maze test. Progesterone, metyrapone or FGIN 1–27 pretreatment increased open arms entries by 31.27% \(F(3,31) = 22.193; P<0.0001\), 42.38% \(F(3,31) = 28.024; P<0.0001\) or 41.15% \(F(3,31) = 22.636; P<0.0001\) and time spent in the open arms by 80.90% \(F(3,31) = 32.724; P<0.0001\), 87.27% \(F(3,31) = 63.574; P<0.0001\) or 84.54% \(F(3,31) = 33.956; P<0.0001\) respectively. The closed

![Fig. 2](image-url) – Influence of ALLO and neurosteroidogenic drugs on effect of etifoxine (25 mg/kg, i.p.) in EPM test on (A) % open arms entries, (B) % time spent in open arms and (C) number of closed arms entries. Rats were injected with metyrapone (50 mg/kg, i.p.) or progesterone (5 mg/kg, i.p.) 30 min before etifoxine (25 mg/kg, i.p.) or vehicle (5 ml/kg, i.p.) or ALLO (0.1 μg/rat, i.c.v.) or FGIN 1–27 (0.5 μg/rat, i.c.v.) 15 min before etifoxine (25 mg/kg, i.p.) or aCSF (5 μl/rat, i.c.v.) treatment. Thirty minutes thereafter rats were subjected to EPM test. Each bar represents mean±S.E.M. of data from 8 rats per group. \(P<0.05\) vs. etifoxine (25 mg/kg, i.p.) (one-way ANOVA followed by Student–Newman–Keuls test).

![Fig. 3](image-url) – Effect of etifoxine (75 mg/kg, i.p.) in the presence of neurosteroid biosynthesis inhibitors in the EPM test on (A) % open arms entries, (B) % time spent in open arms and (C) number of closed arms entries. Rats were injected with finasteride (50×2 mg/kg, s.c. at –4 and –1.5 h) or indomethacin (5 mg/kg, i.p. at –20 min) or trilostane (30 mg/kg, i.p. at –2 h) or PK11195 (15 mg/kg, i.p. at –0.5 h) before etifoxine (75 mg/kg, i.p.) or vehicle (5 ml/kg, i.p.) treatment. Thirty minutes thereafter, rats were subjected to EPM test. Each bar represents mean±S.E.M. of data from 7 rats per group. \(P<0.05\) vs. vehicle; \#\(P<0.05\) vs. etifoxine (one-way ANOVA followed by Student–Newman–Keuls test).
arms entries were not affected by any of these treatments $[F(9,79)=0.8993; P=0.5308]$.

### 2.3. Neurosteroid biosynthesis inhibitors attenuated anxiolytic effect of etifoxine

As shown in Fig. 3, neurosteroid biosynthesis inhibitors like finasteride (50×2 mg/kg, s.c.), trilostane (30 mg/kg, i.p.), indomethacin (5 mg/kg, i.p.) or PK11195 (15 mg/kg, i.p.) at the doses used here per se did not change the entries into and the time spent in the open arms. However, these drugs injected prior to an anxiolytic dose of etifoxine (75 mg/kg, i.p.) significantly reduced the preference for open arms parameters and were not different from vehicle treated control groups. Finasteride, trilostane, indomethacin or PK11195 reduced open arms entries by 25.34% $[F(3,27)=15.877; P<0.0001]$, 21.24% $[F(3,27)=13.345; P<0.0001]$, 22.27% $[F(3,27)=11.685; P<0.0001]$ or 29.46% $[F(3,27)=15.877; P<0.0001]$ and time spent in the open arms by 35.61% $[F(3,27)=31.055; P<0.0001]$, 25.57% $[F(3,27)=28.390; P<0.0001]$, 28.77% $[F(3,27)=31.197; P<0.0001]$ or 23.27% $[F(3,27)=43.264; P<0.0001]$ respectively compared to that of etifoxine (75 mg/kg, i.p.). There was no marked effect of any of these treatments on number of closed arms entries $[F(9,69)=0.5614; P=0.8230]$.

### 2.4. Effect of GABAergic drugs on anxiolytic effect of etifoxine

Muscimol (0.5 mg/kg, i.p.) or bicuculline (0.5 mg/kg, i.p.) at the doses used here per se did not alter any of EPM parameters

![Fig. 4](image_url)  
**Fig. 4** – Influence of GABAergic drugs on effect of etifoxine (25 or 75 mg/kg, i.p.) in EPM test on (A) % open arms entries, (B) % time spent in open arms and (C) number of closed arms entries. Rats were injected with muscimol (0.5 mg/kg, i.p.) 30 min before etifoxine (25 mg/kg, i.p.) or vehicle (5 ml/kg, i.p.). Separate groups were injected with bicuculline (0.5 mg/kg, i.p.) 30 min before etifoxine (75 mg/kg, i.p.) or vehicle (5 ml/kg, i.p.). Rats were subjected to EPM test after 30 min of etifoxine administration. Each bar represents mean±S.E.M. of data from 7 rats per group. *$P<0.05$ vs. vehicle control (5 ml/kg, i.p.) or etifoxine (25 mg/kg, i.p.) or muscimol (0.5 mg/kg, i.p.) or bicuculline (0.5 mg/kg, i.p.); **$P<0.05$ vs. etifoxine (75 mg/kg, i.p.) (one-way ANOVA followed by Student–Newman–Keuls test).
including entries and time spent into open arms as compared to vehicle controls. However, the effect of etifoxine was much greater in muscimol pretreated rats or attenuated in rats that received bicculline injection. Etifoxine (25 mg/kg, i.p.) in the presence of muscimol increased open arms entries by 45.28% \([F(3,27)=11.620; P<0.0001]\) and time spent into the open arms by 85.85% \([F(3,27)=25.241; P<0.0001]\) as compared to only etifoxine treated rats. On the other hand, in bicculline pretreated rats, the effect of etifoxine (75 mg/kg, i.p.) on open arms entries was declined by 25.79% \([F(3,27)=18.138; P<0.0001]\) and time spent into open arms was reduced by 24.78% \([F(3,27)=67.129; P<0.0001]\) as compared to etifoxine alone (Fig. 4).

2.5. Anxiolytic effect of etifoxine in adrenalectomized rats

Bilateral adrenalectomy of rats neither changed the entries (unpaired \(t\) test, \(t=0.1905, df=12, P=0.8521\)) and time spent in the open arms (unpaired \(t\) test, \(t=1.07, df=12, P=0.3041\)) nor did it modify closed arms entries \((P>0.05)\) in EPM test. Anxiolytic dose of etifoxine (75 mg/kg, i.p.) significantly increased the open arm activities in ADX as well as sham-ADX rats. However, no significant difference in open arms behavior \((\% \text{ open arms entries}, t=0.1231, df=12, P=0.9041; \% \text{ time spent in open arms}, t=0.2129, df=12, P=0.8350)\) and closed arms entries \((P>0.05)\) was observed following etifoxine treatment in sham-ADX and ADX groups (Fig. 5).

3. Discussion

In agreement with previous reports (Boissier et al., 1972; Verleye and Gillardin, 2004; Verleye et al., 2005), etifoxine produced a behavioral profile consistent with anxiety reduction. It dose dependently increased the time spent and number of entries into the open arms of EPM. In view of the demonstrable anxiolytic property of etifoxine and neurosteroid ALLO (Bitran et al., 1993; Verleye and Gillardin, 2004; Verleye et al., 2005) and since the neurosteroid biosynthesis inhibitor molecule PK11195 is known to attenuate the etifoxine effect (Schlichter et al., 2000), our results suggest that ALLO plays a significant role in the anxiolytic-like property of etifoxine. Thus, in agreement with the previous report (Verleye et al., 2005), our results suggest that ALLO plays a significant role in the anxiolytic-like effect of etifoxine. In fact, etifoxine enhances the inhibitory GABAergic system involved in the anxiety regulation (Schlichter et al., 2000; Verleye et al., 2001, 2002; Hamon et al., 2003). It acts on GABA\(_A\) receptors at \(\beta_2\) or \(\beta_3\) subunit, a site distinct from benzodiazepines (Sigel and Buhr, 1997; Korpi et al., 2002) or neurosteroids (Sigel and Buhr, 1997; Hamon et al., 2003). Thus the increased anxiolytic effect of etifoxine in presence of ALLO or other neurosteroidogenic drugs could be due to enhanced GABA\(_A\) receptor function by their direct allosteric effects (Verleye et al., 2001). Alternatively, etifoxine is also reported to increase GABAergic neurotransmission by stimulating mitochondrial-type benzodiazepine receptors leading to augmented neurosteroid synthesis (Schlichter et al., 2000; Verleye et al., 2005). Thus, there appeared to be a correlation between etifoxine treatment and neurosteroid in the brain.

To strengthen the above hypothesis, we administered several neurosteroidogenic enzyme inhibitors prior to etifoxine. For example, triostane inhibits \(3\alpha\)-hydroxysteroid dehydrogenase \((3\alpha\text{-HSD})\) to prevent the conversion of pregnalone to progesterone in the brain (Potts et al., 1978; Korneyev et al., 1993), finasteride, a \(5\alpha\)-reductase inhibitor, that prevents the conversion of progesterone to \(5\alpha\)-dihydropregesterone \((5\alpha\text{-DHP})\) (Burton-Jones et al., 1999; Concas et al., 1998; Kokate et al., 1999; VanDoren et al., 2000) and indomethacin, that inhibits enzyme \(3\alpha\)-hydroxysteroid oxidoreductase \((3\alpha\text{-HSOR})\), thereby preventing conversion of \(5\alpha\)-DHP to ALLO (Beyer et al., 1999). Interestingly, the anxiolytic effect of etifoxine was significantly inhibited by these agents. Moreover, PK11195, an antagonist of PBRs that restrict the transport of cholesterol to inner membrane of mitochondria (Schlichter et al., 2000), also significantly blocked the anxiolytic effect of etifoxine. Thus, in agreement with the previous report (Verleye et al., 2005), the effect of etifoxine in presence of ALLO or other neurosteroidogenic drugs could be due to enhanced GABA\(_A\) receptor function by their direct allosteric effects. In fact, etifoxine enhances the inhibitory GABAergic system involved in the anxiety regulation (Schlichter et al., 2000; Verleye et al., 2001, 2002; Hamon et al., 2003). It acts on GABA\(_A\) receptors at \(\beta_2\) or \(\beta_3\) subunit, a site distinct from benzodiazepines (Sigel and Buhr, 1997; Korpi et al., 2002) or neurosteroids (Sigel and Buhr, 1997; Hamon et al., 2003). Thus the increased anxiolytic effect of etifoxine in presence of ALLO or other neurosteroidogenic drugs could be due to enhanced GABA\(_A\) receptor function by their direct allosteric effects (Verleye et al., 2001). Alternatively, etifoxine is also reported to increase GABAergic neurotransmission by stimulating mitochondrial-type benzodiazepine receptors leading to augmented neurosteroid synthesis (Schlichter et al., 2000; Verleye et al., 2005). Thus, there appeared to be a correlation between etifoxine treatment and neurosteroid in the brain.

To strengthen the above hypothesis, we administered several neurosteroidogenic enzyme inhibitors prior to etifoxine. For example, triostane inhibits \(3\alpha\)-hydroxysteroid dehydrogenase \((3\alpha\text{-HSD})\) to prevent the conversion of pregnalone to progesterone in the brain (Potts et al., 1978; Korneyev et al., 1993), finasteride, a \(5\alpha\)-reductase inhibitor, that prevents the conversion of progesterone to \(5\alpha\)-dihydropregesterone \((5\alpha\text{-DHP})\) (Burton-Jones et al., 1999; Concas et al., 1998; Kokate et al., 1999; VanDoren et al., 2000) and indomethacin, that inhibits enzyme \(3\alpha\)-hydroxysteroid oxidoreductase \((3\alpha\text{-HSOR})\), thereby preventing conversion of \(5\alpha\)-DHP to ALLO (Beyer et al., 1999). Interestingly, the anxiolytic effect of etifoxine was significantly inhibited by these agents. Moreover, PK11195, an antagonist of PBRs that restrict the transport of cholesterol to inner membrane of mitochondria (Schlichter et al., 2000), also significantly blocked the anxiolytic effect of etifoxine. Thus, in agreement with the previous report (Verleye et al., 2005), our results suggest that ALLO plays a significant role in the anxiolytic-like effect of etifoxine. However, etifoxine and ALLO bind to distinct recognition sites (Verleye et al., 2001), their enhancing action may be an additive effect. Furthermore, the involvement of other neurosteroids in the etifoxine-induced anxiolysis cannot be ignored.

In addition, GABA\(_A\) receptor agonist, muscimol potentiated, its antagonist bicculline inhibited the anxiolytic action of etifoxine. This finding supports the earlier studies which suggest that etifoxine potentiates GABA\(_A\) receptor function and facilitates GABAergic transmission (Verleye et al., 1999, 2001, 2002; Schlichter et al., 2000). We may recall that the anxiolytic effect of etifoxine was attenuated by antagonist of PBRs and neurosteroid biosynthesis inhibitors, indicating the indirect action of etifoxine on GABA\(_A\) receptors via production of neurosteroids (Schlichter et al., 2000; Verleye et al., 2001, 2005). Furthermore, to assess the absolute contribution of central neurosteroids and not the adrenal steroids in the etifoxine induced anxiolysis, the experiments were also carried out in bilateral adrenalectomized rats. Surprisingly, the anxiolytic-like effect of etifoxine was not altered by adrenalectomy, thus ruling out the involvement of steroids from peripheral source, at least...
adrenal steroids. However, this must be interpreted with caution since gonads were not ablated. Thus, it is possible that increased production of neurosteroids particularly ALLO by etifoxine may contribute to its anxiolytic effect.

4. Experimental procedures

4.1. Subjects

Adult Sprague-Dawley male rats (220–260 g body weight and 90–110 days old) were housed five per cage (cage size 640×410×250 mm, acrylic) before surgical procedures, thereafter they were housed individually. Animals were maintained at 24±1 °C under 12:12 h light/dark cycle (lights on 07:00–19:00 h) with rodent chow (Lipton, India) and water ad libitum. Rats were housed individually and brought to the experimental room 12 h prior treatments to minimize non-specific stress-induced steroid fluctuations. The experimental procedures were in strict accordance with the guidelines approved by the Institutional Experimental Animal Ethical Committee of Rashtrasant Tukadoji Maharaj Nagpur University, Department of Pharmaceutical Sciences, Nagpur, India. Animals were naive to drug treatment and experimentation at the beginning of each study. Each experimental group had a separate set of seven to nine animals.

4.2. Drugs and administration

ALLO, progesterone (ALLO precursor), muscimol (GABA<sub>A</sub> receptor agonist), bicuculline (GABA<sub>A</sub> receptor antagonist), FGIn 1–27 (mitochondrial diazepam binding inhibitor receptor (MDR) agonist), PK11195 (PBR antagonist) and metyrapone (11β-hydroxylase inhibitor) were purchased from RBI, USA. Etifoxine (Biocodex Laboratories, France), finasteride (Dr. Reddy’s Laboratories, India), trilostane (Sanofi Winthrop Development Center, UK) and indomethacin (Zim Laboratories, India) were received as gift samples. Etifoxine, ALLO, progesterone, metyrapone, FGIn 1–27, muscimol, bicuculline, finasteride, trilostane, indomethacin and PK11195 were dissolved in 2-hydroxypropyl-β-cyclodextrin (45% w/v) solution and diluted with 0.9% saline or for intracerebroventricular (i.c.v.) administration with artificial cerebrospinal fluid (aCSF, composition: NaCl 0.2 M, NaH₂CO₃ 0.02 M, HCl 0.5 mM, KH₂PO₄ 1.2 mM, CaCl₂ 1.8 mM, MgCl₂ 0.5 mM, Na₂SO₄ 5.8 mM and d-glucose 0.12 M) was administered over a period of 1 min into the right lateral ventricle. The injection cannula was left in place for further 1 min before being slowly withdrawn to avoid back flow. On completion of the experiments, 5 μl dilute India ink was injected through the cannula and animals were immediately euthanized by overdose of thiopentone sodium. Post-mortem examination of the brain sections was undertaken to confirm the correct placement of cannula and the data of only those animals with uniform distribution of ink into the ventricles were used for statistical analysis. The animals that showed incorrect placement of guide cannula or neurological deficits (<15%) were excluded from the study.

4.3. Intracerebroventricular (i.c.v.) administration

As reported from our laboratory earlier (Khisti et al., 2002; Ugale et al., 2004b; Hirani et al., 2005; Kokare et al., 2006), rats were anesthetized with thiopentone sodium (45 mg/kg, i.p.) (Abbott Pharmaceuticals, Mumbai, MS, India) and fixed in a stereotaxic frame (David Kopf, USA). A stainless-steel guide cannula (24 gauge, C316DC/Spc, internal diameter 0.29 mm; outer diameter 0.56 mm) (Plastics One Inc., Roanoke, VA, USA) was implanted aseptically into the right lateral ventricle (coordinates from Paxinos and Watson, 1998; posterior –0.8 mm; lateral from midline +1.2 mm and ventral –3.5 mm; relative to bregma). The guide cannula was secured in place by dental cement (Dental Products of India, Mumbai) affixed to two mounting screws (Plastics One). A stainless-steel dummy cannula (28 gauge, C316DC/Spc, o.d., 0.25 mm) (Plastics One) was used to occlude the guide cannula when not in use. Animals were then housed singly and allowed for a week to recover from surgery before any experimentation. During this 7-day period, animals were also habituated to testing environment by transferring to experimental room and twice-daily handling. Injections were made using microliter syringe (Hamilton, Nevada, USA) connected to stainless-steel internal cannula (31 gauge, C316I/Spc, i.d., 0.12 mm; o.d., 0.25 mm) (Plastics One) by polyethylene tubing (PE-10, i.d., 0.28 mm; o.d., 0.61 mm). A volume of 5 μl was administered over a period of 1 min into the right lateral ventricle. The injection cannula was left in place for further 1 min before being slowly withdrawn to avoid back flow. On the day of experiment animals were shifted to behavioral room 1 h prior to testing to facilitate adaptation. Rats were placed singly in the center square of the maze, head facing one of the open arms and allowed to explore for 5 min. The number of entries into and time spent in each arm were recorded by an observer blind to the treatment. Arm entry was registered as placing all four paws of the animal on it. The maze was wiped clean with damp cotton and dried after testing each rat. Anxiogenic or anxiolytic effects were assessed based on the frequency of entries as well as time spent in the open arms (Pellow et al., 1985). Increase in time spent as well as frequency of entries in the open arms relative to control animals was considered as an anxiolytic behavior. Separate groups of animals were used for each treatment and each subject tested was given a single 5 min trial or tested once only to avoid ‘one trial tolerance’ to drug effect (Bertoglio and Carobrez, 2002).

4.5. Drug treatments

4.5.1. Dose related effect of etifoxine and ALLO in EPM test

Separate groups (n=7–9) of rats were injected with different doses of etifoxine (25–75 mg/kg, i.p.) or ALLO (0.1–1.0 μg/rat, i.c.v.) and 30 min after i.p. or 15 min after i.c.v. injection, individual rat
was subjected to EPM test for 5 min as described above. The control animals were given an equivalent volume (5 ml/kg, i.p. or 5 μl/rat, i.c.v.) of vehicle.

4.5.2. Influence of ALLO and neurosteroidogenic drugs on anxiolytic effect of etifoxine

In these experiments, the effect of ALLO and the drugs that increase endogenous brain neurosteroid levels on etifoxine-induced anxiolysis was assessed. Different groups (n=8) of rats were injected with progesterone (5 mg/kg, i.p.) or 11-α-hydroxylation inhibitor metyrapone (50 mg/kg, i.p.) 30 min prior to etifoxine (25 mg/kg, i.p.) or vehicle (5 ml/kg, i.p.) treatment. Other groups of rats were injected with neurosteroid ALLO (0.1 μg/rat, i.c.v.) or MDR agonist FGIN 1–27 (0.5 μg/rat, i.c.v.) 15 min prior to etifoxine (25 mg/kg, i.p.) or vehicle (5 μl/rat, i.c.v.) treatment and 30 min thereafter, rats were subjected individually to EPM test for 5 min.

4.5.3. Effect of neurosteroid biosynthesis inhibitors on etifoxine-induced anxiolysis

These experiments were conducted to determine the influence of reduced neurosteroid brain content on etifoxine-induced anxiolysis. We administered various neurosteroid biosynthesis inhibitors before etifoxine injection. The doses of inhibitors used and their injection intervals were selected on the basis of existing literature. Neurosteroid biosynthesis inhibitors such as 5α-reductase types I and II inhibitor (Blument-Jones et al., 1999; Concass et al., 1998; Kokate et al., 1999; VanDoren et al., 2000) finasteride (50 ± 2 mg/kg, s.c. at −4 and −1.5 h), 3α-hydroxysteroid oxidoreductase (3α-HSOR) inhibitor (Beyer et al., 1999) indomethacin (5 mg/kg, i.p. at −20 min), 3β-hydroxysteroid dehydrogenase (3β-HSD) inhibitor (Potts et al., 1978; Korneyev et al., 1993) trilostane (30 mg/kg, i.p. at −2 h) or PBR antagonist (Schlichter et al., 2000) PK11195 (15 mg/kg, i.p. at −0.5 h) were injected before etifoxine (75 mg/kg, i.p.) or vehicle (5 ml/kg, i.p.; n=7 per group). Individual rat was then subjected for 5 min to EPM test after 30 min of etifoxine or vehicle treatment.

4.5.4. Effect of GABAergic drugs on anxiolytic effect of etifoxine

Rats (n=7) were injected with the GABA_A receptor agonist muscimol (0.5 mg/kg, i.p.) 30 min prior to a fixed subeffective dose (25 mg/kg, i.p.) of etifoxine. On the other hand, GABA_A receptor antagonist bicuculline (0.5 mg/kg, i.p.) was administered at the same time interval prior to effective dose (75 mg/kg, i.p.) of etifoxine. Control groups treated with vehicle (5 ml/kg, i.p.) were also assessed during these studies. Following an interval of 30 min, individual rat was allowed to explore EPM for a 5 min test session.

4.5.5. Etifoxine anxiolysis in adrenalectomized rats

These experiments were performed to investigate the contribution of adrenal steroids in the anxiolytic effect of etifoxine. Rats were surgically, bilaterally adrenalectomized (ADX) or sham-operated (sham-ADX) under thiopentone sodium (45 mg/kg, i.p.) anesthesia using dorsal approach. After surgery, rats were provided with 1% sucrose solution in 0.9% saline daily and were allowed to recover for 1 week before being subjected to experiments. ADX or sham-ADX rats (n=7) were treated with vehicle (5 ml/kg, i.p.) or etifoxine (75 mg/kg, i.p.) and 30 min later subjected to the EPM test.

4.6. Statistical analysis

Data are expressed as means ± S.E.M. and analyzed by one-way analysis of variance (ANOVA). Significant interactions were assessed by post hoc Dunnett’s or Student–Newman–Keuls test. Unpaired t test was used to analyze the data of adrenalectomy experiment. A value of P < 0.05 was considered as statistically significant for all the treatments.

Acknowledgments

The authors express gratitude to Departement de Pharmacologie, Laboratoires Biocodex, Zac de Mercieres, Chemin d’Armancourt, 60200 Compiegne, France for providing the gift sample of etifoxine.

REFERENCES


Corpechot, C., Robel, P., Axelson, M., Sjovall, J., Baulieu, E.E., 1981. Characterization and measurement of


Sharma, A.N., Chopde, C.T., Hirani, K., Kokare, D.M., Ugsale, R.R., 2007. Chronic progesterone treatment augments while dehydroepiandrosterone sulphate prevents tolerance to...


