Differential effects of the 18-kDa translocator protein (TSPO) ligand etifoxine on steroidogenesis in rat brain, plasma and steroidogenic glands: Pharmacodynamic studies

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ABSTRACT

Etifoxine is indicated in humans for treating anxiety. In rodents, besides its anxiolytic-like properties, it has recently shown neuroprotective and neuroregenerative activities. It acts by enhancing GABA<sub>A</sub> receptor function and by stimulating acute steroid biosynthesis via the activation of the 18-kDa translocator protein. However, the regulatory action of etifoxine on steroid production is not well characterized. In this work, we performed dose-response, acute and chronic time-course experiments on the effects of intraperitoneal injections of etifoxine on steroid levels in adult male rat brain and plasma analyzed by gas chromatography-mass spectrometry. Concentrations of pregnenolone, progesterone and its 5α-reduced metabolites were significantly increased in both tissues in response to 25 and 50 mg/kg of etifoxine, as compared with vehicle controls, and reached maximal values at 0.5–1 h post-injection. Daily injections of etifoxine (50 mg/kg, 15 days) kept them increased at day 15. Comparisons between steroidogenic tissues revealed that 1 h after 50 mg/kg of etifoxine treatment, levels of pregnenolone, progesterone and corticosterone were highest in adrenal glands and markedly increased together with their reduced metabolites. They were also increased by etifoxine in brain and plasma, but not in testis except for corticosterone and its metabolites. In contrast, testosterone level was significantly decreased in testis while with its 5α-reduced metabolites, it was unchanged in brain. Results demonstrate that the modulation of steroid concentrations by etifoxine is dependent on the type of steroid and on the steroidogenic organ. They further suggest that adrenal steroids upregulated by etifoxine make an important contribution to the steroids present in brain. This work provides a precise and complete view of steroids regulated by etifoxine that could be useful in therapeutic research.

1. Introduction

The mitochondrial 18-kDa translocator protein (TSPO), originally named the peripheral benzodiazepine receptor due to its ability to bind diazepam with high affinity (Lacapere and Papadopoulos, 2003), is a cholesterol binding protein located in the outer mitochondrial membrane for steroid synthesis (Papadopoulos et al., 2006; Papadopoulos and Miller, 2012). TSPO is ubiquitously distributed with predominant expression in steroid-synthesizing tissues such as gonads, adrenal glands and brain. In addition to its role in cholesterol transport and steroidogenesis, TSPO can regulate several other biological functions such as mitochondrial respiration, cell proliferation, apoptosis, oxidative stress, and microglial activation (Veenman et al., 2007; Corsi et al., 2008; Caballerio et al., 2013; Wang et al., 2014; Santoro et al., 2016). It is also a biomarker for inflammation, traumatic brain injury, neurodegeneration and psychiatric disorders (Rupprecht et al., 2010). The role of TSPO in the regulation of cellular bioenergetics is reinforced by two complementary approaches using Tsop gene deletion (Banati et al., 2014) and Tsop gene insertion (Liu et al., 2017). Noteworthy, studies in Tspo knockout mice report normal phenotype, thus challenging previously postulated TSPO functions and TSPO ligand selectivity and mechanisms of action. This apparent discrepancy may however be explained by the adaptive modifications resulting from a functional network operating with TSPO (Gut et al., 2015).

Etifoxine chlorydrate (EFX) (Stresam®; Biocodex Laboratories) is a...
drug clinically approved for the treatment of psychosomatic manifestations of anxiety. This benzoxazine compound displays anxiolytic effects similar to classical benzodiazepines but without some of their negative side effects (Nguyen et al., 2006; Stein et al., 2015). Thus, at an anxiolytic dose in humans, EFX shows limited psychomotor and mnesic side-effects, and causes no physical dependence after treatment cessation (Micallef et al., 2001; Nguyen et al., 2006; Choi and Kim, 2015; Stein et al., 2015). In rodents, EFX (50 mg/kg, i.p.) also exhibits marked anxiolytic-like properties, reduces physiological manifestations of stress (Verleye and Gillardin, 2004; Verleye et al., 2005; Ugale et al., 2007). Beside its traditional benefits on neuropsychological symptoms, EFX has recently received increasing attention as a remarkable neuroregenerative and neuroprotective molecule. In rodents, EFX (50 mg/kg, i.p.) improves peripheral nerve regeneration together with the recovery of motor and sensory motor functions (Girard et al., 2008; Zhou et al., 2014). It also exerts strong anti-hyperalgesic, anti-aldolonic and anti-inflammatory properties in a model of peripheral mononeuropathy in rats (Aouad et al., 2014a). It protects against inflammatory demyelination in a mouse model of multiple sclerosis (Daugherty et al., 2013; Ravikumar et al., 2016) and reduces neuroinflammation in rat model of traumatic brain injury (Simon-O’Brien et al., 2016).

Several in vitro and in vivo studies in rats have suggested that the anxiolytic effect of EFX involve a double action on TSPO and on central GABA_A receptors (GABA_A-Rs), thus potentiating GABAergic inhibitory neurotransmission. Firstly, EFX directly binds to GABA_A-Rs containing a β2/β3 subunit at a site close to the chloride channel and distinct from the benzodiazepine binding site (Schlichter et al., 2000; Hamon et al., 2003). Secondly, EFX indirectly increases brain neuroactive steroid levels, such as allopregnanolone by activating TSPO (Verleye et al., 2005; Ugale et al., 2007). Allopregnanolone is an endogenous 3α-hydroxy-5α-reduced metabolite of progesterone (3α,5α-THP) that modulates positively GABA_A receptors in the nervous system (Lambert et al., 1995). Thus, allopregnanolone shares many neuropsychological characteristics with EFX. Interestingly, early reports have indicated that the potentiation of GABA_A receptor chloride channel by 3α,5α-THP can be blocked by its 3β-stereoisomer 3β,5α-THP which is nevertheless inactive on these receptors (Wang et al., 1999). 3β,5α-THP is now recognized as a member of a novel class of GABA_A receptor modulating steroid antagonists (GAMSA) (Johansson et al., 2016), thus offering new therapeutic approaches for enhancement of brain excitability.

Local production of allopregnanolone mediates the anxiolytic effects of EFX in rats (Ugale et al., 2007). The promotion of peripheral nerve recovery and the reduction of neuropathic pain symptoms by EFX are also mediated by an increase in fast GABAergic inhibition and local production of allopregnanolone (Poisbeau et al., 2014). From these studies, it appears that promoting endogenous steroid synthesis by the administration of EFX might be beneficial in several diseases of the nervous system and may represent an alternative approach for steroid therapeutics. However, there is few published data on the pharmacological regulation of steroid synthesis by EFX (Verleye et al., 2005; Aouad et al., 2014b; Ravikumar et al., 2016). Within this context, it is crucial to characterize steroidogenic pathways and tissues influenced by EFX.

The present work was designed to examine in detail the regulation of steroidogenesis by EFX in adult male rats. In particular, levels of pregnenolone (PREG), progesterone (PROG) and its 5α-reduced metabolites, testosterone and its 5α-reduced metabolites, and androstenedione (ADIONE) were measured in both brain and plasma, in dose-response and kinetics experiments. As brain steroids may arise from local synthesis and/or peripheral endocrine glands, steroid profiles were established in brain, plasma, testes and adrenal glands of both control and EFX-treated rats. We analyzed the concentrations of PREG, PROG, deoxycorticosterone (DOC) and corticosterone, testosterone and 17β-estradiol (E2) and their main metabolites, by using a sensitive, selective and precise GC/MS analytical procedure. This is the most reliable technology to identify and quantify low amounts of steroids in tissues and biological fluids (Meffre et al., 2007; Hertig et al., 2010).

2. Methods

2.1. Animals

All procedures were carried out in strict accordance with the recommendations of the EU Directive 2010/63/EU for animal experiments in an approved animal facility (approval 94-043-13).

Male adult Sprague-Dawley rats (Janvier Laboratories; Genêt-Št Isle, France) 8–9 weeks of age and weighing 300–350 g were used. They were four per cage in an animal facility with controlled temperature (22 °C ± 1 °C) and humidity (50% ± 5%). They were maintained on a 12 h light-dark cycle and had access to food and water ad libitum. Upon arrival, they were acclimatized to house facility conditions for 5–7 days before study initiation. They were then submitted to a habituation/handling period with the principal authorized investigator.

2.2. Etifoxine treatments and experimental groups

Etifoxine (EFX, 2-ethyl-amino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride, batch number 302) was provided by Biocodex Laboratories (Gentilly, France). It was dissolved in NaCl 0.9% containing 1% Tween 80 (vol/vol) (Vehicle, VEH). The mixture was freshly prepared prior to the injections and maintained with constant agitation. Separate groups of animals were administered with EFX or VEH by the intra-peritoneal (i.p.) route using a volume of 5 ml/kg. For each study, rats were randomly assigned to the EFX or VEH-control groups. Steroid concentrations were determined blind to the animal treatments. A further investigator raised the blind and performed the statistical analyses.

For the dose-response study, rats received increasing doses of EFX, 12.5 mg/kg, 25 mg/kg or 50 mg/kg by i.p. injections (n = 8 rats/group). Rats treated with VEH served as controls. Steroids were analyzed in the brain and plasma at 1 h post-treatment. This time interval was chosen based on previous results showing a marked and significant increase of PREG in the brain (Verleye et al., 2005).

For the acute experiments, rats received i.p. injections of EFX 50 mg/kg or VEH (n = 6 rats/group). Steroids were analyzed in the brain and plasma at 0.25, 0.5, 1, 2 and 24 h post-administration.

For the chronic experiments, EFX 50 mg/kg or VEH was i.p. administered once daily during 15 days. Steroids were analyzed in brain and plasma 1 h post-administration at days 1, 3, 7 and 15 of the experiment (n = 8 rats/group).

The steroid profiling was determined in brain, plasma, testis and adrenal glands, 1 h after an acute i.p. administration of EFX (50 mg/kg i.p.) or VEH (n = 8 rats/group).

2.3. Steroid profiling by gas chromatography/mass spectrometry (GC/MS)

Blood was collected in heparinized plastic tubes and plasma was prepared and stored at −80 °C. Forebrains (with olfactory bulbs), testis and adrenals were harvested, frozen in liquid nitrogen, weighed and stored at −80 °C.

A panel of steroids was identified and quantified simultaneously in individual tissues by GC/MS as previously described (Gonzalez et al., 2016). Briefly, steroids were first extracted from forebrains (687–972 mg), testis (1323–1591 mg), adrenals (32–65 mg) and plasma (1 ml) with 10 vols of methanol (MeOH). Adrenal glands, plasmas and aliquots corresponding to 400 mg of forebrains and testis extracts were withdrawn. The following internal standards were added into the extracts for steroid quantification: 2 ng of 3H,5α-dihydroprogesterone (DHP) (CDN Isotopes, Sainte Foy la Grande, France) for 5α-/β-DHP and 5α-/β-androstenedione (5α-/β-ADIONE), 2 ng of 3H-testosterone for testosterone, 2 ng of 3H-17β-estradiol (17β-E2) for 17β-E2 and estrone (E1), 2 ng of epieticholanolone (Steroidals, Newport, Rhode Island) for