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Involvement of the $GABA_A$ receptor α subunit in the mode of action of etifoxine

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ABSTRACT

Etifoxine (EFX) is a non-benzodiazepine psychoactive drug which exhibits anxiolytic effects through a dual mechanism, by directly binding to $GABA_A$ receptors ($GABA_ARs$) and to the mitochondrial 18-kDa translocator protein, resulting in the potentiation of the GABAergic function. The β subunit subtype plays a key role in the EFX-$GABA_AR$ interaction, however this does not explain the anxiolytic effects of this drug. Here, we combined behavioral and electrophysiological experiments to challenge the role of the $GABA_AR$ α subunit in the EFX mode of action. After single administrations of anxiolytic doses (25-50 mg/kg, intraperitoneal), EFX did not induce any neurological nor locomotor impairments, unlike the benzodiazepine bromazepam (0.5-1 mg/kg, intraperitoneal). We established the EFX pharmacological profile on heteropentameric $GABA_ARs$ constructed with α1 to α6 subunit expressed in *Xenopus* oocyte. Unlike what is known for benzodiazepines, neither the γ nor δ subunits influenced EFX-mediated potentiation of GABA-evoked currents. EFX acted first as a partial agonist on α2β3γ2S, α3β3γ2S, α6β3γ2S and α6β3δ $GABA_ARs$, but not on α1β3γ2S, α4β3γ2S, α4β3δ nor α5β3γ2S $GABA_ARs$. Moreover, EFX exhibited much higher positive allosteric modulation towards α2β3γ2S, α3β3γ2S and α6β3γ2S than for α1β3γ2S, α4β3γ2S and α5β3γ2S $GABA_ARs$. At 20 μM, corresponding to brain concentration at anxiolytic doses, EFX increased GABA potency to the highest extent for α3β3γ2S $GABA_ARs$. We built a docking model of EFX on α3β3γ2S $GABA_ARs$, which is consistent with a binding site located between α and β subunits in the extracellular domain. In conclusion, EFX preferentially potentiates α2β3γ2S and α3β3γ2S $GABA_ARs$, which might support its advantageous anxiolytic/sedative balance.

Chemical compounds studied in this article: etifoxine (PubChem CID: 171544), bromazepam (PubChem CID: 2441), diazepam (PubChem CID: 3016)

Keywords: etifoxine; $GABA_A$ receptors; α subunit; anxiolysis; behavioral pharmacology; EFX-binding mode

Highlights

- We investigated the influence of α subunits of $GABA_AR$ on the mode of action of etifoxine, a non-benzodiazepine compound.
- Etifoxine induces anxiolysis without locomotion impairment and sedation in mice.
- Etifoxine strongly potentiates α3β3γ2S and moderately α2β3γ2S and α6β3γ2S $GABA_ARs$ compared to other $GABA_ARs$.
- A docking model of EFX with α3β3γ2S $GABA_AR$ reveals a binding site at the α/β interface, close to the GABA-binding pocket.

Abbreviations

α(1-6)$GABA_ARs$, α1 to α6 subunit-containing $GABA_A$ receptors; BZD, benzodiazepine; BZP, bromazepam; DZP, diazepam; EFX, etifoxine; $GABA_AR$, $GABA_A$ receptors; PAM, positive allosteric modulator; TEVC, two-electrode voltage-clamp

1. Introduction

$GABA_AR$s are heteropentameric membrane proteins that belong to the cys-loop ligand-gated ion channel superfamily [1]. They are permeant to chloride ions in response to GABA and decrease neuronal excitability through membrane hyperpolarization. To date, 19 mammalian $GABA_AR$ subunits have been described and cloned (α1-6, β1-3, γ1-3, δ, ε, π, θ, ϱ1-3) [1]. The putative combination of these subunits provides a large heterogeneity of $GABA_AR$s, with a stoichiometry of 2α, 2β and a complementary subunit (mainly γ or δ). The contribution of $GABA_AR$s to fast or slow neuronal inhibition depends on their stoichiometry, their tissue distribution and their synaptic or extrasynaptic location. The most frequent assembly of synaptic receptors is α(1-3)β(1-3)γ(1-3), whereas extrasynaptic receptors dominantly contain α4 or α6 with β(1-3) and δ, or α5, β(1-3) and γ2 [1,2]. These differences in stoichiometry and distribution support their different neurophysiological functions and pharmacological properties [3,4].

$GABA_AR$s are targeted by benzodiazepines (BZDs) and other drugs for the treatment of anxiety, epilepsy and sleep disorders [5,6]. BZDs act as positive allosteric modulators (PAMs) of $GABA_AR$s by binding to a site at the interface between γ2 subunit and α subunits [7]. Classical BZDs, such as diazepam (DZP, Fig. 1), bromazepam (BZP, Fig. 1) and lorazepam, exhibit similar pharmacological profile in behavioral tests [8,9] and display poor selectivity over $GABA_AR$s which contain α1, or α2 or α3 or α5 (α1$GABA_AR$, α2$GABA_AR$, α3$GABA_AR$, or α5$GABA_AR$) [10], which explains their undesirable effects, including withdrawal symptoms, sedation, amnesia, cognitive impairments and aggressiveness. Indeed, α1$GABA_AR$s are associated with sedation, BZD addiction, anterograde amnesia, anticonvulsant activity and cortical plasticity [10-12]. α2$GABA_AR$s and α3$GABA_AR$s have been linked to anxiolysis, antihyperalgesia and myorelaxation [13-15]. α5$GABA_AR$s are believed to be correlated to sedation, cognitive impairments and more recently, anxiolysis [14-17].

Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride, EFX, Fig. 1) is a non-BZD compound that exhibits anxiolytic and anticonvulsant effects in rodents [18] and is used for the treatment of anxiety-related disorders in humans [19,20]. EFX also displays anti-hyperalgesic and anti-inflammatory properties in different animal models [21,22]. Both *in vitro* and *in vivo* studies in rats suggested that the anxiolytic effects of EFX involve a dual mechanism of action, by directly binding to central $GABA_A$Rs and to the mitochondrial 18-kDa translocator protein (TSPO) with, as a result, potentiation of the GABAergic function [23,24]. Indeed, it has been shown that EFX activates TSPO through a direct binding and consecutively stimulates the synthesis of neurosteroids, such as, allopregnanolone, which act as PAMs of $GABA_A$Rs [25-27]. Although the affinity of EFX for $GABA_A$Rs was twice higher than the one for TSPO (K_i of 6.1 μM vs K_i of 12.7 μM), the predominance of one of the effect over the other, i.e. direct $GABA_A$Rs binding or through TSPO activation, in mediating its anxiolytic effect, is still debated [25,28,29].

The importance of the β subunit in the mode of action of EFX on $GABA_A$Rs has been clearly evidenced [24]. Constitutively-open homopentameric β$GABA_A$Rs are inhibited by EFX. In addition, α1$GABA_A$Rs and α2$GABA_A$Rs embedding β2 or β3 are more sensitive to EFX than α1$GABA_A$Rs and α2$GABA_A$Rs with β1. These data underline the importance of the β subunit in the EFX-$GABA_A$R interaction and for EFX-potentiation of GABA-induced currents of heteromeric $GABA_A$Rs. However, homopentameric β2$GABA_A$Rs are less sensitive to EFX than homopentameric β1$GABA_A$Rs [24], suggesting that the nature of the α subunit might also play a role in EFX-$GABA_A$Rs interaction. In addition, at anxiolytic doses, EFX has no sedative effects nor locomotion impairment in humans [19] or rodents [30] and this could hardly be explained by the equal potency of EFX on α2$GABA_A$R and α1$GABA_A$R since the latter is associated with sedation [10,12]. Thus, we hypothesized that the pharmacological profile of EFX reflects different sensitivities of all α subtypes containing-$GABA_A$Rs.

In this study, we first compared EFX and BZP in anxiolysis, sedation and locomotor impairment behavioral tests in acute conditions, in mice. The pharmacological effects of both EFX

and BZP already appear after a single administration [8,9,29,30]. Here, we determined the anxiolytic doses of EFX and their possible influences on motor performance and arousal. We then assessed the impact of α subunit isoforms on the effects of EFX on GABA-evoked currents. We characterized the pharmacological profile of EFX on murine synaptic $GABA_A$Rs (α1β3γ2S, α2β3γ2S, α3β3γ2S, α4β3γ2S and α6β3γ2S) and extrasynaptic $GABA_A$Rs (α5β3γ2S, α4β3δ and α6β3δ) using electrophysiology. This pharmacological study was completed with a 3D model, showing the interaction between EFX and $GABA_A$Rs. Our results demonstrate that the EFX mode of action involves both α and β, but not γ or δ, subunits.

2. Materials and methods

2.1 Ethical statements

All animal procedures were carried out in accordance with the European Community council directive 2010/63/EU for the care and use of laboratory animals and were approved by our respective local ethical committees (N°CEEA.45 and N°CEEA.72 for mice and N°CEEA.2012.68 for *Xenopus*, https://www.ceea-paysdelaloire.com/) in addition to the French Ministry of Agriculture (authorization N°B49071 and N° 02200.02). The NC3R's ARRIVE guidelines were followed in the conduct and reporting of all experiments using animals.

2.2 Animal care and conditioning

Experiments were carried out using 7- to 9-week-old Balb/cByJ mice (25-30 g) purchased from Charles River Laboratory (Les Oncins, France). Ten mice per translucent polypropylene cage (internal dimensions in mm: 375 x 375 x 180, L x W x H) were housed under standard laboratory conditions (22 ± 2° C, 12-h light/dark cycle, lights on at 7:00 AM) with food (AO4, SAFE, France) and tap water available *ad libitum*. No less than one week of rest followed their arrival. Mice were habituated to the testing room at least 60 min before performing any behavioral evaluation. All tests occurred between 9:00 AM and 3:00 PM. The behavioral tests were performed by two well-trained experimenters, who remained unaware of the administered treatment. In addition, all equipment was wiped with 70% ethanol between animals to erase the olfactory stimuli. All experiments were performed in a randomized manner. Single administrations of EFX (12.5-150 mg/kg, expressed as hydrochloride salt) or BZP (0.25-1 mg/kg) were given by the intraperitoneal (IP) route, 30 min before each test, except in the stress-induced hyperthermia test in which the compounds were administered 60 min before the test. Studies have shown that both compounds have a similar profile with plasma peak at 15-30 min [25,31-33]. The control animals received an equivalent volume of vehicle (0.9% NaCl, 1% tween 80 (v/v)). One male C57Bl/6N mouse was used for the cloning experiments. This

mouse had been included in a control group (not treated) from a previous protocol in which mice were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Euthanasia was performed using CO_2 (3 ml/min, 4 min). Adult female *Xenopus laevis* were purchased from CRB (Rennes, France) and had been bred in the laboratory in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the European Community. Oocytes were harvested from mature female *Xenopus laevis* frogs under 0.15% tricaine anaesthesia. All animals recovered after 2-3 h. Every female is operated every three months, not less. A single female was used no more than 5 times.

2. 3 Compounds

EFX hydrochloride (batch 653, Biocodex, France), and BZP (batch 5788, Francochim, France) (Fig. 1) were suspended in vehicle and administered (IP) in a volume of 10 ml/kg of body weight. For electrophysiology, EFX and DZP (batch 105F0451, Sigma, France) were dissolved in dimethylsulfoxide (DMSO) resulting in a maximal concentration of 0.1% DMSO, for oocyte perfusion (control experiments were performed to demonstrate no effect of DMSO). All other reagents and solvents were obtained from Sigma-Aldrich Merck (Saint-Louis, MO, USA) or Thermo Fisher Scientific (Waltham, Massachusetts, USA).

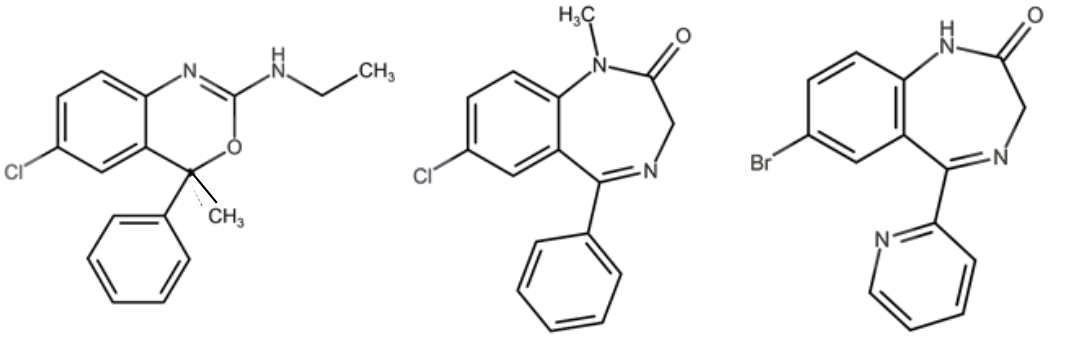


Fig. 1. Structure of the positive allosteric modulators of $GABA_A$Rs used in this study. The 2D structure of EFX (PubChem CID: 171544), DZP (PubChem CID: 3016) and BZP (PubChem CID: 2441) are illustrated.

2.4 Anxiety-related behavioral assessment

Stress-induced hyperthermia (SIH) is defined as the increase in body temperature observed when a subject is exposed to an external stressor [34]. The day preceding the experiment, the animals were isolated in smaller cages (dimensions: 265x160x140 mm). The body temperature (± 0.1°C) of one singly housed mouse was measured twice via a rectal probe (YSI n°423; 2 mm diameter) coupled to a thermometer (Letica-Temp812 model-Italia) at an interval of 10 min. The rectal temperature measurement procedure (handling, insertion of the probe) constitutes the stressor. Drugs were injected 60 min before the first measurement (T1), followed by a second temperature measurement 10 min later (T2). Methodological experiments have shown that the optimal conditions for drug testing are found with an injection-test interval of 60 min or longer to avoid residual effects of the injection procedure. Indeed, using a 60 min-injection-stress interval results in a hyperthermic response comparable to animals that are not injected [35,36]. The reduction of SIH ($\Delta T = T2-T1$) is considered to reflect an anxiolytic-like effect [34-36]. Defensive burying after novel object exploration is a behavior that can be elicited in rodents in response to aversive or new stimuli [37]. Mice were singly housed in smaller cages (see above) with a sawdust depth of 2.5 cm the day before the test. Each mouse was confronted with an unfamiliar object (4x4x6 cm; aluminium) introduced in the centre of the cage. The time of contact or exploring the object (snout pointing toward the object at a distance < 1 cm with or without burying) was recorded for 10 min. The object was cleaned with alcohol (10%) between each trial. This behavioral approach reveals an anxiety-like or fear state and its suppression is associated with a reduction of the anxiety-like behavior [37].

2.5 Spontaneous locomotor activity

Testing was conducted in a quiet room under a light level of approximately 400 lux. The motor activity cages (dimensions: 265x160x140 mm) were made of clear plastic and were changed between each animal; these cages contained a minimum amount of sawdust. The locomotor activity was

measured by infra-red beam interruptions that were counted by a control unit (OptaVarimex, Columbus, Ohio, USA). The sensitivity of this unit was set so that walking (horizontal activity) and rearing (vertical activity) were measured. The beam breaks corresponding to spontaneous locomotor activity were measured for 15 min.

2.6 Rotarod performance

The rotarod test assesses motor performance by measuring the capacity of mice to remain on a 3-cm-diameter rod revolving 16 rpm (model 7600; Ugo Basile, Comerio, Italy). The mice were trained to walk on the rotarod until they could complete three consecutive 120 s sessions without falling off the rod. Twenty-four hours later, selected animals were treated with drugs before being challenged. The rotarod performance time was measured three times, up to 120 s, and the mean was adopted as the performance time for each animal.

2.7 $GABA_AR$ subunit cDNA expression vectors

pRK7 plasmids containing cDNAs encoding rat α2 subunits were a kind gift from Professor Harmut Lüddens (University of Mainz, Mainz, Germany). The $GABA_AR$ α2 subunits from rat and mouse are identical in amino acid sequence. pGW1 (=pRK5) plasmids containing cDNAs encoding mouse β3 and γ2S subunits were kindly provided by Professor Steven J. Moss (Tufts University, Boston, USA). The cDNAs encoding the α3, α4, α5, α6 and δ subunits used in this work were cloned in mouse brain as described below.

2.8 RNA extraction, RT-PCR and cloning of full-length cDNAs encoding α3-6 and δ subunits

The brain was dissected from a male C57Bl/6N mouse for RNA extraction and purification. Total RNA was then extracted using TRIzol® Reagent (Ozyme/Biogentex, France). First strand cDNAs were synthesised from 5 μg of total RNA using SuperScriptTM III First-Strand Synthesis System Super Mix (Invitrogen, USA) in the presence of oligo (dT)20, according to the manufacturer's

instructions. cDNAs encoding α3-6 and δ subunits were amplified using gene-specific primer pairs encompassing each ORF (Supplemental Table 1) and high-fidelity thermostable DNA polymerase (Advantage 2 Proofreading Polymerase kit, Clontech, Saint-Germain-en-Laye, France). cDNAs fragments were purified with the Nucleospin PCR Cleanup Kit (Macherey-Nagel, Hoerdt, Germany) and were subsequently cloned into PCR® 4 TOPO® (Invitrogen). Each clone was sequenced twice on both strands using universal sense and reverse primers by GATC Biotech (Konstanz, Germany). Sequence analyses were performed using BioEdit sequence analysis software. To transfer α3-6 and δ subunits ORFs into the pRK5 expression vector, we adapted the ligase-free method for directional cloning [38]. Plasmids and cDNA inserts were separately prepared by PCR using the proof reading polymerase, KOD DNA polymerase (Merck Millipore, Fontenay sous Bois, France). To generate sticky-end cDNAs, two individual PCR reactions were performed, PCR1 and PCR2, with gene-specific primers containing short overhangs that allow annealing with the complementary overhangs of the plasmid. pRK5 plasmid was modified to clone the $GABA_AR$ subunit ORFs flanked at their 5' end by alfalfa mosaic virus (AMV) coat protein (RNA 4) and at their 3'end by 3′ -untranslated regions (UTRs) from the Xenopus β-globin gene (3UTRXBG). The combination of both UTRs has been shown to improve expression in both oocytes and mammalian cells [39]. First, a fusion of AMV and 3'UTRXBG was constructed and cloned into pRK5 between EcoRI and XbaI. The resulting modified vector (pRK5-5AMV-3UTRXBG) was used as a template for two individual PCRs with the following pair primers: 5'-TAAACCAGCCTCAAGAACACCCGA-3' with 5' GGTGGAAGTATTTGAAAGAAAATTAAAAATA-3' (PCR1), and 5'-AAGCTTGATCTGGTTACCACTAAACC-3' with 5'-AAAATTAAAAATAAAAACGAATTCAATCGATA-3' (PCR2). PCR1 and PCR2 products were purified and mixed in T4 ligase buffer. To generate cDNA with sticky ends, the amplicons were subjected to melting and reannealing, as previously described [38]. Inserts containing $GABA_AR$ subunit ORFs were also prepared in two individual PCRs (LIC-PCR1 and 2) with gene-specific

primer pairs (see Table S1 in supplementary data). Sticky-end inserts were obtained as described for the plasmid preparations. For each construct, sticky-ends plasmid and $GABA_A$ cDNA preparation were assembled in T4 ligase buffer and incubated for 2 h at 22°C. One to two microliters of this assemblage were used to transform chemically competent E. coli cells (DG1, Eurogentec, Seraing, Belgium). The resulting clones were sequenced as described above.

2.9 Expression of $GABA_A$Rs in Xenopus oocytes

Adult female *Xenopus laevis* (CRB, Rennes, France) were anaesthetized in ice-cold water with 0.15% Tricaine (3-aminobenzoic acid ethyl ester, Sigma). Ovarian lobes were collected and washed in standard oocyte saline (SOS containing 100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4). Stage V-VI oocytes were partially defolliculated by enzymatic treatment with 2 mg/ml collagenase (type IA, Sigma) in Ca^{2+}-free SOS for 60 min. To express functional $GABA_AR$, cDNA mixtures were directly injected into the nucleus (animal pole) of individual defolliculated oocytes in different volumes of DNA solution at a concentration of 50 ng/μl using a nanoinjector (Drummond Nanoject) (see Table S2 for receptor stoichiometry and DNA quantity injected). Following injection, the oocytes were kept at 18°C in SOS supplemented with gentamycin (50 μg/ml), penicillin (100 UI/ml), streptomycin (100 μg/ml), and sodium pyruvate (2.5 mM). The incubation medium was replaced every two days. Oocytes were incubated 1 to 2 days after DNA injection, depending on the $GABA_AR$ subtype.

2.10 Electrophysiological recordings

Injected oocytes were tested for $GABA_AR$ expression, at a holding potential of -60 mV using a two-electrode voltage clamp amplifier (TEV-200, Dagan Corporation, Minneapolis, USA). Digidata 1440A interface (Axon CNS Molecular Devices, California, USA) and pCLAMP 10 software (Axon CNS Molecular Devices) were used for acquisition. Cells were continuously superfused with standard oocyte saline (SOS) at room temperature and were challenged with drugs in SOS. Electrodes were

filled with 1 M KCl / 2 M K acetate and had typical resistances of 0.5–2 MΩ in SOS. Drugs were perfused at a flow rate close to ~4 ml/min. EFX and DZP were applied for 2 min before co-application of GABA at EC_{10}, (determined for each $GABA_AR$ subtype, see Table S2 in supplementary data) until the current response peaked. EFX was tested at concentrations ranging from 2 to 100 μM, corresponding to its clinical use. Between the two applications, the oocytes were washed in SOS for 10-15 min to ensure full recovery from receptor desensitization (see Fig. 4A inbox). To control whether GABA-evoked currents were mediated by ternary α1-6β3γ2S$GABA_AR$s, control experiments were performed using SOS containing 10 μM Zn^{2+} to inhibit binary $GABA_AR$s [40]. Data were analysed using pCLAMP 10 software. Data are expressed as the mean ± SEM of 6-10 oocytes generated from at least two collections. Concentration-effect relationships were analysed using the following equation: $Y=Ymin + (Ymax-Ymin)/(1+10^{((LogEC50-X)\cdot nH)})$, where X is the concentration of EFX, Ymin and Ymax are the minimum and highest responses, and nH is the Hill coefficient.

2.11 Molecular model design

The template chosen for homology modelling was the recently solved structure of a $GABA_AR$ (pdb code 4COF). The sequences of the human α2, β3 and γ2S $GABA_AR$ subunits were aligned with those of the template (β3) using T-Coffee software [41]. The model was then prepared by homology modeling using Modeler version 9.5 software [42] with default settings. One hundred models were prepared, and the best model, according to the Discrete Optimized Protein Energy function (DOPE), was selected. Side chains in the models were improved with Scwrl4 [43]. The whole model was then improved with CHARMM [44,45]. Disulfide bridges formed between neighbouring cysteines both in the 'Cys-loop' and between the M1 and M3 transmembrane helices in α and γ subunits, as recently proposed [45]. The model was then subjected to minimization with decreasing harmonic potential.

2.12 Docking

The docking had been performed with AutoDock Vina [46]. The ligands and proteins were prepared with prepare_ligand4.py and prepare_receptor4.py scripts, respectively. The side chains of amino-acids in the binding site were made flexible (α3: Ser257 Ser258 and β3 Gln66, Tyr87, Gln89, Tyr91). Each ligand was docked 100 times in a large cube of 30 Å in each dimension. Fig. 7 was prepared with PyMOL (DeLano W.L. (2010) The PyMOL Molecular Graphics System, version 1.6, Schrodinger, LLC, New York).

2.13 Data analysis and statistics

Data are presented as the mean ± SEM. Behavioral data were analysed by one-way ANOVA followed by Dunnett's post-hoc test for comparison with the vehicle group. In cases in which the two conditions (normality of the data and equality of variances) were not fulfilled, the non-parametric Kruskal-Wallis procedure was used, followed by the post-hoc Dunn's test to evaluate the statistical significance between the vehicle and treated groups. All test analyses were carried out by observers who were blinded to the experimental procedures. Sample sizes (number of animals in the behavioral studies) were not predetermined by a statistical method. Each behavioral experiment group included at least 10 animals and this sample size needed to detect significant effects was based on experience from previous studies. Significance tests between groups in the electrophysiological studies were performed using variance analysis (one-way ANOVA) followed by Tukey's post-hoc test for comparison of all groups or the non-parametric Mann and Whitney test when appropriate. Concerning the electrophysiological experiments, we compiled data from different batches of oocytes and we excluded data, in case of potential drift (> 0.6 mV) after pulling out the electrodes from the oocytes and when current amplitudes were <10 nA or > 2 µA. GraphPad Prism 7.02 (GraphPad Software, San Diego, USA) was used for all graphs and statistical analyses. Differences with $p<0.05$ were considered significant (* for $p<0.05$, ** for $p<0.01$, *** for $p<0.001$, **** for $p<0.0001$).

3. Results

3.1 Anxiolytic effects of EFX

Previous studies have shown that EFX exhibits anxiolytic effects using conventional behavioral tests such as elevated plus maze and dark-light box tests [33,47]. Here, we evaluated the effect of EFX on stress and anxiety-related behaviors (stress-induced hyperthermia and novel object exploration) to determine and confirm its anxiolytic doses in comparison with BZP. In non-treated animals, handling stress resulted in a rise in body temperature close to 1°C (Fig. 2A to D). BZP significantly lowered body temperature in animals at 1 mg/kg dose, before handling (T0) revealing hypothermia ($H(3)=11.343$, $p=0.010$, then $p<0.05$, Dunn's test) (Fig. 2A). BZP dose-dependently prevented stress-induced hyperthermia ($F_{3,41}=18.290$, $p<0.001$) (Fig. 2B). Compared to the vehicle-treated animals, BZP was effective at doses of 0.5 and 1 mg/kg ($p<0.05$, Dunnett's test). EFX also induced changes in body temperature but without hypothermia at the highest dose (50 mg/kg) compared with control animals ($F_{3,41}=2.269$, $p=0.095$) (Fig. 2C). As observed for BZP, the temperature increase was dose-dependently prevented by EFX ($H(3)=20.072$, $p<0.001$) with a significant effect at 25 and 50 mg/kg dose ($p<0.05$, Dunn's test) (Fig. 2D). The anxiolytic effects of BZP and EFX were also assessed by evaluating the behavioral approach in the presence of an unfamiliar object. The duration of contact with a novel object was significantly decreased in animals treated with BZP ($H(3)=15.304$, $p=0.002$) from the dose of 1 mg/kg ($p<0.05$, Dunn's test) (Fig. 2E). The same was observed with EFX ($H(3)=17.536$, $p<0.001$), with a significant effect observed at doses of 25 and 50 mg/kg compared with the control animals ($p<0.05$, Dunn's test) (Fig. 2F). In conclusion, these two different behavioral tests led to similar anxiolytic doses of EFX (25-50 mg/kg, IP).

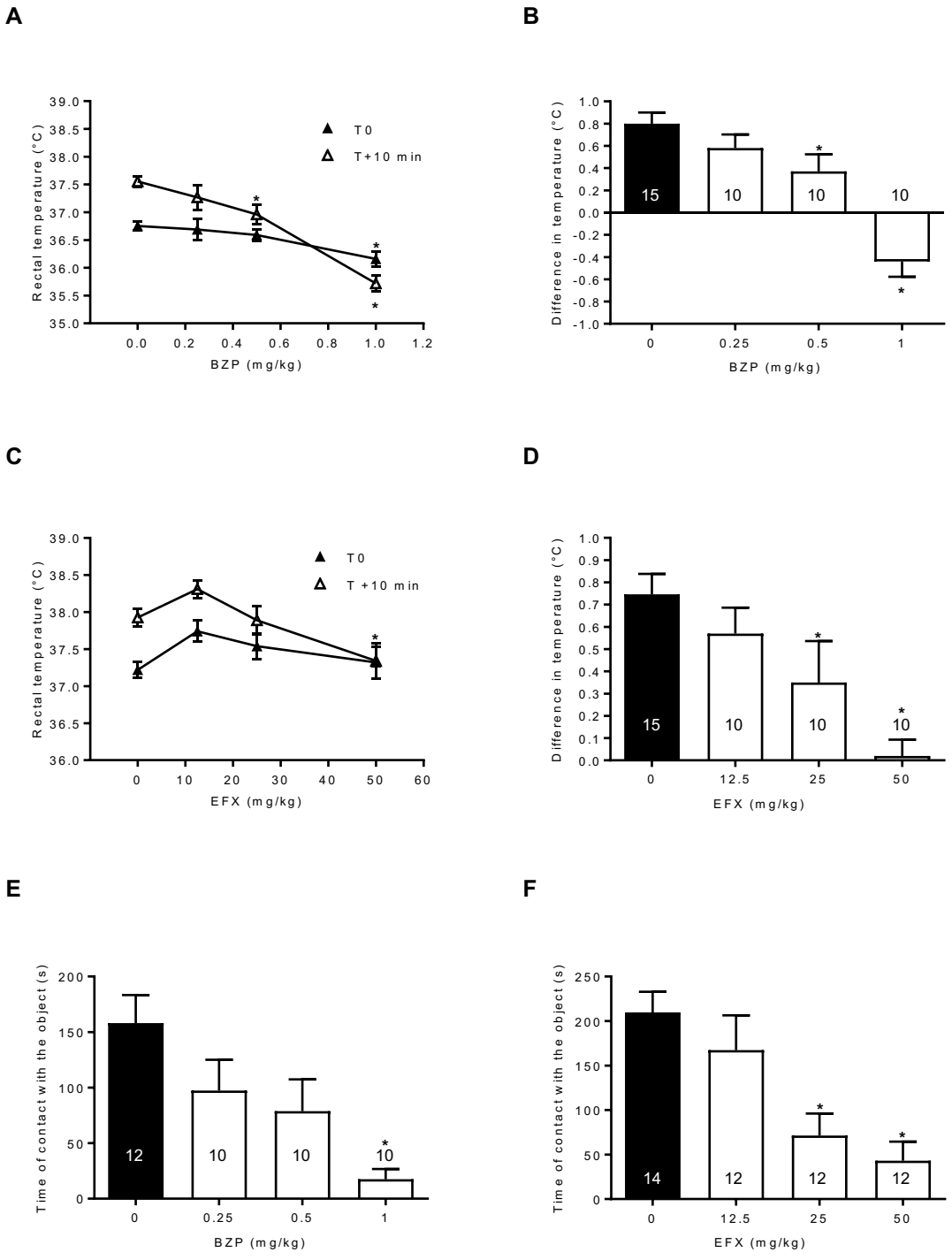


Fig. 2. Comparison of the effects of EFX and BZP on anxiety-related behaviors in mice. (A-D) The graphs show the evolution of the mean rectal temperature (± SEM) at T0 and T0+10 min after treatment with vehicle (dose 0) or BZP (A) or EFX (C) through IP route, 60 min before the first temperature measurement at T0. Histograms represent the mean (± SEM) of the difference of the rectal temperature measured at T0 and T+10 min in the same mouse after treatment with vehicle or BZP (B) or EFX (D) at the indicated doses. *p<0.05 compared with the vehicle group (Dunn test). (E,F) Histograms illustrate the mean time (± SEM) of contact with an unfamiliar object after IP administration of BZP (E) or EFX (F). *p<0.05 compared with the respective vehicle groups (dose 0). (see "data analysis and statistics" in the Material and Methods section). Animal numbers are indicated inside the bars.

370 *3.2 Motor performance assessment*

371 We then compared the impact of EFX and BZP on locomotion performance and motor
372 coordination (Fig. 3). As illustrated in Fig. 3, BZP (IP route) decreased spontaneous locomotor
373 activity ($H(3)=8.229$, $p=0.042$). These effects were statistically significant for the 1 mg/kg dose
374 ($p<0.05$; Dunn's test) (Fig. 3A). EFX (IP route) decreased spontaneous locomotor activity with a
375 significant difference at 100 mg/kg ($H(4)=9.633$, $p=0.047$) compared to control animals ($p<0.05$,
376 Dunn's test) (Fig. 3B). BZP reduced the time on the rotarod ($H(3)=9.167$, $p=0.027$), with a significant
377 effect at the 1 mg/kg dose ($p<0.05$, Dunn's test) (Fig. 3C). EFX was devoid of any effect up to the
378 100 mg/kg dose and affected motor coordination at the 150 mg/kg dose compared with control
379 animals ($H(5)=19.006$, $p=0.002$ then $p<0.05$, Dunn's test) (Fig. 3D). In conclusion, BZP triggers
380 motor impairments at anxiolytic doses (1 mg/kg, IP), while EFX exhibits no locomotor effects at
381 efficient anxiolytic doses (25 to 50 mg/kg, IP).

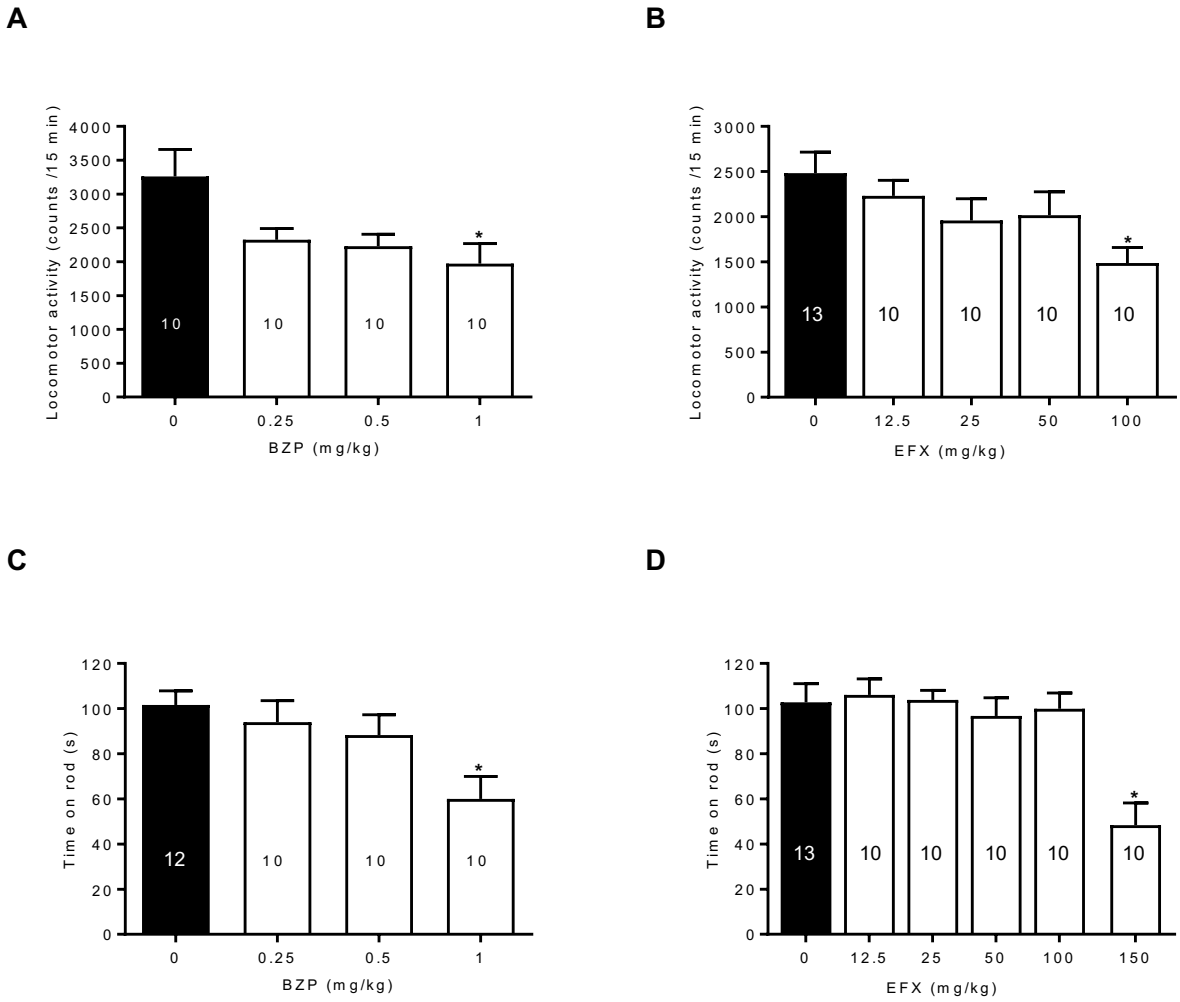


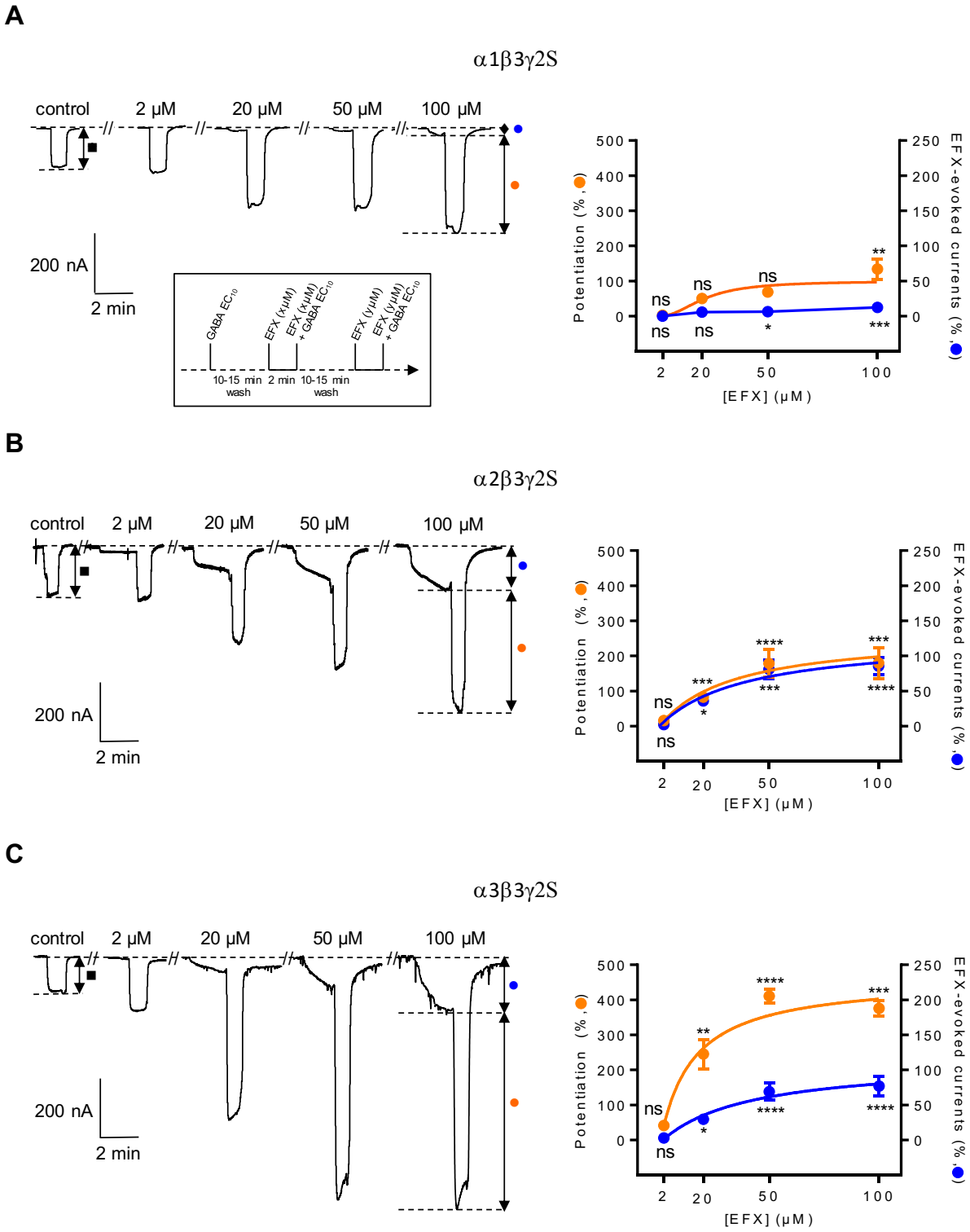
Fig. 3. Comparison of BZP and EFX on locomotor activity and motor coordination. (A, B) Spontaneous locomotor activities were assayed in the actimeter test in mice after IP injections of BZP (A) or EFX (B). The bars represent the means ± SEM of the number of infrared beam interruptions over15 min. (C,D) Histograms illustrate the time on the rod (mean ± SEM) after injection of BZP (C) or EFX (D). Animal numbers are indicated inside the bars. *p <0.05 compared with the respective vehicle groups (see "data analysis and statistics" in the Material and Methods section).

3.3 EFX effects on GABA currents depends on α subunit isoforms

Because the distribution of $GABA_AR$ α subunits within the CNS is heterogeneous and contributes to their receptor functions, we next investigated the involvement of α subunits in the EFX mode of action using electrophysiology. To achieve this goal, we compared the effects of EFX (2 to 100 μM) on GABA-induced currents elicited by α1$GABA_ARs$, α2$GABA_ARs$, α3$GABA_ARs$, α4$GABA_ARs$, α5$GABA_ARs$ or α6$GABA_ARs$, containing $\beta_3$together with γ2S or δ, when appropriate.

We first challenged EFX effects on GABA-currents elicited by synaptic α1β3γ2S, α2β3γ2S and α3β3γ2S $GABA_ARs$ expressed in *Xenopus* oocytes (Fig. 4). We observed that EFX displays both

agonist and potentiating effects as previously described [24]. The agonist effects of EFX were revealed by its perfusion before co-application with GABA at EC_{10} (Table S2). EFX exhibits almost no agonist effects on α1β3γ2S $GABA_A$Rs (12.4 ± 5.8 and 23.7 ± 10.4 % of GABA EC_{10} at 50 and 100 μM EFX, respectively) (Fig. 4A). In contrast, comparable agonist effects were observed with α2β3γ2S $GABA_A$Rs (171 ± 24.9 % of GABA EC_{10}, at 100 μM EFX, Fig. 4B) and α3β3γ2S $GABA_A$Rs (153.6 ± 27.3% of GABA EC_{10}, at 100 μM EFX, Fig. 4C). In comparison with GABA, EFX exerted weaker agonist effects (~100 fold less efficient). For these three receptors, EFX potentiation of GABA EC_{10}-evoked currents was dose-dependent, reaching a plateau at 50 μM (Fig. 4). At this concentration, EFX induced a potentiation of GABA EC_{10}-evoked currents by 68.9 ± 11.6%, 160.3 ± 40.2% and 410.7 ± 20.2% of α1β3γ2S, α2β3γ2S and α3β3γ2S, respectively. The potentiating effects induced by EFX from 2 to 100 μM, was ~2.4-6.0-fold stronger ($p<0.05$) with α3β3γ2S than with α1β3γ2S and α2β3γ2S $GABA_A$Rs. Taking in account both agonist and potentiating effects, α1β3γ2S $GABA_A$Rs are found much less sensitive to EFX than α2β3γ2S and α3β3γ2S $GABA_A$Rs.



409 **Fig. 4.** Effects of EFX on GABA-activated currents mediated by three synaptic $GABA_A$Rs (α1β3γ2S, α2β3γ2S and
410 α3β3γ2S). EFX effects were investigated by TEVC in *Xenopus* oocytes expressing α1β3γ2S (A), α2β3γ2S (B) and
411 α3β3γ2S $GABA_A$Rs (C). (A-C) Increasing concentrations of EFX (2, 20, 50 and 100 μM) were applied 2 min before co-
412 application of GABA at EC_{10}(inbox). The amplitudes of EFX-evoked currents (●) were normalized to the amplitude of
413 control currents (■) obtained with GABA alone at EC_{10}. The potentiation effects of EFX was determined as the percentage
414 increase of EC_{10}-GABA current amplitudes (●). Left panel, GABA EC_{10}-induced representative currents are illustrated,
415 showing the partial agonist and positive modulatory effects of EFX. Right panel, data points (mean ± SEM of 6-11 oocytes
416 from at least two different animals) were fitted by non-linear regression to the Hill equation with variable slope using
417 GraphPad Prism 7. Statistical analyses were performed using one-way ANOVA tests followed by Tukey's post-hoc
418 correction (comparison with data obtained at 2 μM, *p<0.05, **p <0.01; ***p <0.001; ****p <0.0001, ns: not significant).

Based on pharmacokinetic data [30], we estimated that 20 μM matches the concentration of free EFX in the mouse brain after injection of anxiolytic doses (25-50 mg/kg, Fig. 2). Thus, we compared agonist and potentiating effects of 20 μM EFX on α(1-3) β3γ2S $GABA_A$Rs (Fig. 5A and B). EFX (20 μM) acts as a partial agonist on α2$GABA_A$Rs and α3$GABA_A$Rs, while these agonist effects are not significant on α1$GABA_A$Rs (Fig. 5A). As for the potentiating effects, α3$GABA_A$Rs were much more sensitive to EFX (245.6 ± 41.8%) than α1 (50.1 ± 12.5%) and α2 (101.7 ± 12.2% increase) -containing $GABA_A$Rs (Fig. 5B). In the synaptic cleft the GABA concentration rapidly rises up to the millimolar range [48], we thus compared the GABA concentration-response relationships in the presence and absence of 20 μM EFX at α1$GABA_A$Rs, α2$GABA_A$Rs and α3$GABA_A$Rs (Fig. 5C, Table 1). EFX induced a decrease of GABA EC_{50} with α1$GABA_A$Rs and α2$GABA_A$Rs in a similar extent (~4-fold). In contrast, the GABA potency on α3$GABA_A$Rs was increased by 20.6 (Fig. 5C). Taken together, our electrophysiological data show that, EFX behaves as a selective PAM of α3$GABA_A$Rs at concentration equivalent to anxiolytic doses.

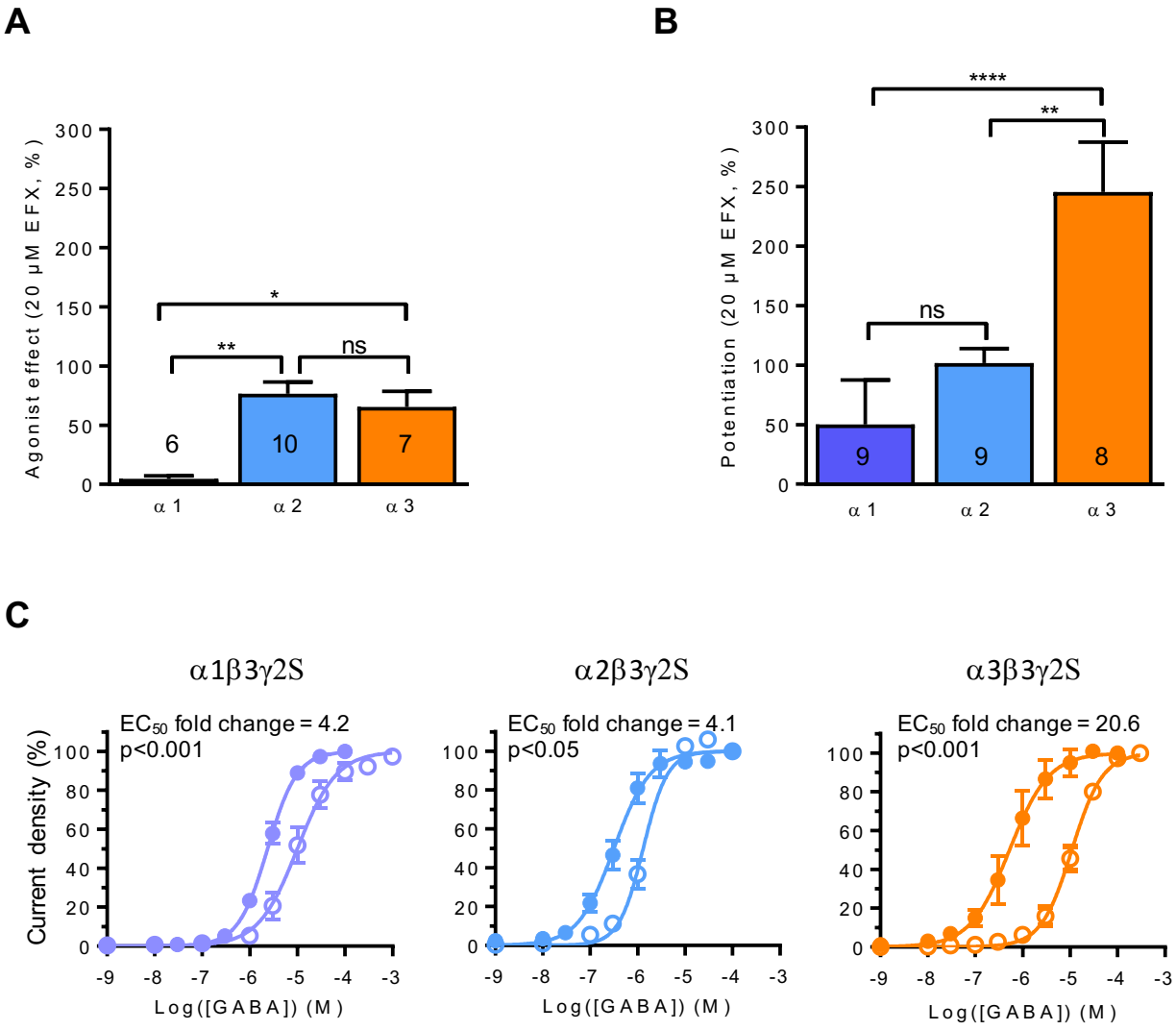


Fig. 5. Pharmacological profile of EFX over α(1-3)β3γ2S GABA$_A$Rs. Comparison of EFX effects at 20 μM corresponding to anxiolytic doses. Detailed analysis of agonist (A) and potentiating (B) effects of 20 μM EFX on α1β3γ2S, α2β3γ2S and α3β3γ2S GABA$_A$Rs. One-way ANOVA followed by Tukey post-hoc test was used for the analysis ((*p<0.05, **p <0.01; ***p <0.001; ****p <0.0001). The number of recorded oocytes is indicated above or inside the bars. (C) Concentration-response curves of GABA-evoked currents in the absence (open circles) and presence (close circles) of 20 μM EFX. Statistical analyses were performed using a non-parametric Mann & Whitney unpaired t-test. All data are expressed as the mean ± SEM (n ≥ 6).

Table 1

Parameters of the GABA concentration-response relationship at α1β3γ2S, α2β3γ2S and α3β3γ2S GABA$_A$Rs modulated by 20 μM EFX.

	control		+ 20 µM EFX	
GABA$_A$R subtype	EC_{50} (µM)	nH	EC_{50} (µM)	nH
α1β3γ2S	9.99 ± 0.96	1.06 ± 0.09	2.37 ± 0.11	1.41 ± 0.08
α2β3γ2S	1.31 ± 0.12	1.69 ± 0.28	0.32 ± 0.03	1.14 ± 0.01
α3β3γ2S	10.89 ± 0.61	1.34 ± 0.09	0.53 ± 0.08	1.05 ± 0.15

The concentration-response relationships were analyzed using the Hill-Langmuir equation with variable slope. nH : Hill slope. The data are mean ± SEM of at least two independent experiments.

445 Next, we tested EFX on synaptic α4β3γ2S $GABA_A$Rs and extrasynaptic α4β3δ $GABA_A$Rs. In
446 both cases, EFX did not exhibit any agonist effects nor significantly potentiate GABA-induced
447 currents, even at high concentrations (Fig. 6A,B). Conversely, DZP at 2 μM enhanced GABA currents
448 elicited by α4β3γ2S $GABA_A$Rs, as previously reported [49], but it did not affect α4β3δ $GABA_A$Rs
449 (Fig. 6A,B). The extrasynaptic α5β3γ2S $GABA_A$Rs appeared to be also insensitive to EFX at low
450 concentrations (2 and 20 μM), and weakly sensitive to EFX at 50 μM (54.26 ± 29.19%) (Fig. 6C and
451 D). At 100 μM, EFX effects were significantly increased but did not reach a plateau (212.0 ± 31.97%).
452 The α6 subunit was expressed with γ or δ in accordance with the native $GABA_A$R composition in the
453 cerebellum [50]. Similar agonist effects were observed with α6β3γ2S $GABA_A$Rs (76.9 ± 20.3% of
454 GABA EC_{10}, at 100 μM EFX,) and α6β3δ $GABA_A$Rs (46.8 % of GABA EC_{10}, at 100 μM EFX) (p=0.23,
455 Mann and Whitney test) (Fig. 6E,F) as seen with α2$GABA_A$Rs and α3$GABA_A$Rs (Fig. 4B,C).
456 Moreover, the PAM effects of EFX revealed equal sensitivities of synaptic α6β3γ2S$GABA_A$Rs and
457 extrasynaptic α6β3δ $GABA_A$Rs. EFX-potentiation of GABA-evoked currents reached a plateau at 50
458 μM EFX (170.5 ± 48.4% increase for α6β3γ2S $GABA_A$Rs and 143.6 ± 22.3% for α6β3δ $GABA_A$Rs,
459 p>0.99, Mann and Whitney test) in accordance with a specific concentration-dependent mode of
460 action (Fig. 6E and F).

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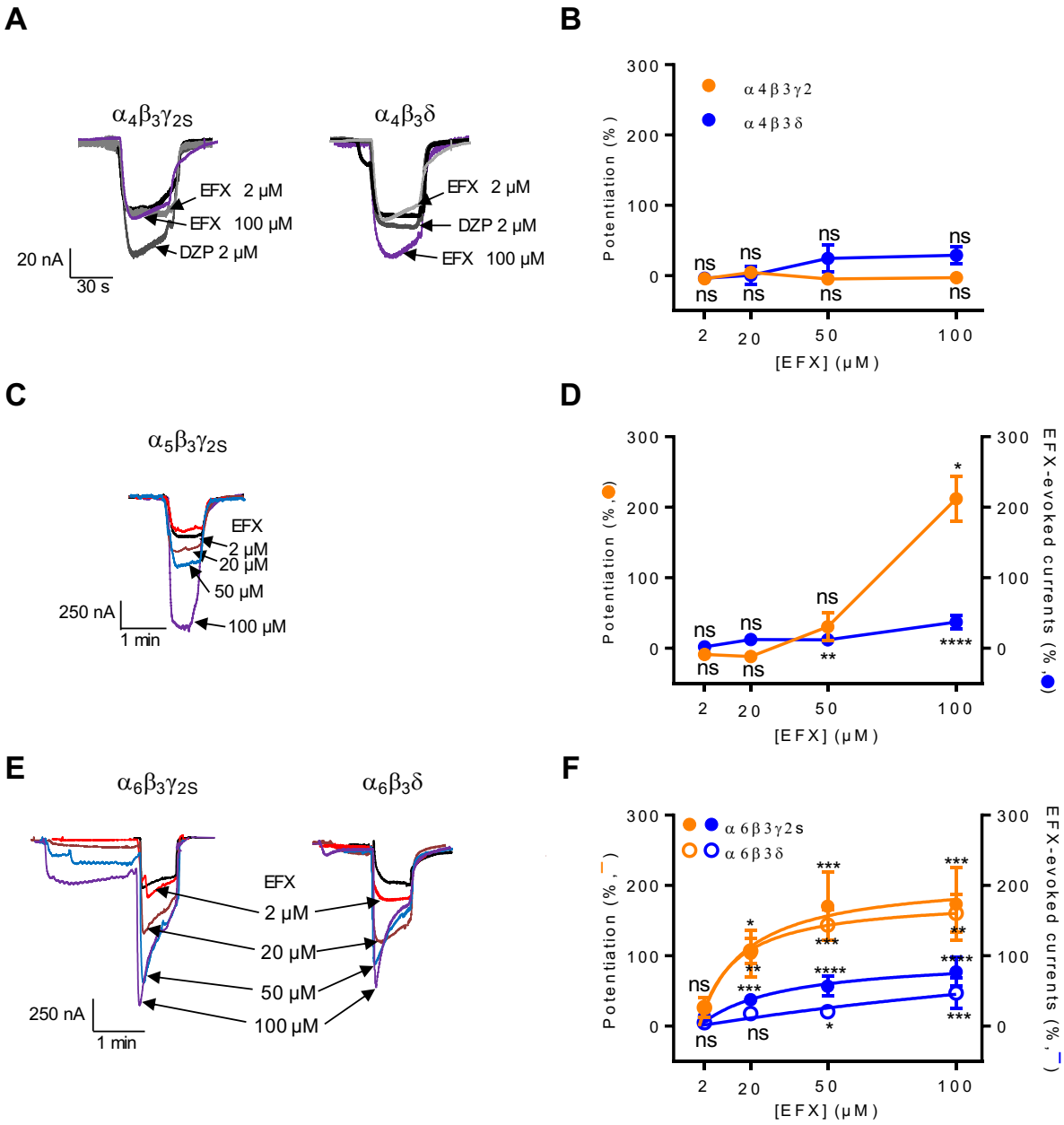

462 **Fig. 6.** Effects of EFX on GABA EC_{10}-activated currents mediated by synaptic (α4β3γ2S and α6 β3γ2S) and extrasynaptic
463 (α4β3δ, α5β3γ2S and α6β3δ) $GABA_A$Rs. (A) Superimposed current traces evoked by GABA EC_{10} in a representative cell
464 expressing α4β3γ2S or α4β3δ $GABA_A$Rs in the absence (black traces) or presence of DZP (2 μM) or EFX. (B) Graphs
465 illustrating the mean (± SEM) EFX potentiation of GABA EC_{10}-activated currents mediated by synaptic and extrasynaptic
466 α4$GABA_A$Rs. (C) Current traces evoked by GABA EC_{10} in a representative oocyte expressing extrasynaptic α5β3γ2S
467 $GABA_A$Rs in the absence (black trace) or presence of EFX (2 to 100 μM). (D) Graphs showing the mean (± SEM) EFX
468 potentiation of GABA EC_{10}-evoked currents. Statistical analyses were performed by one-way ANOVA followed by Tukey
469 post-hoc test (*p<0.05, **p <0.01; ***p <0.001; ****p <0.0001, ns: not significant). (E) Current traces evoked by GABA
470 EC_{10} with synaptic α6β3γ2S and extrasynaptic α6β3δ $GABA_A$Rs in the absence (black traces) or presence of increasing
471 concentrations of EFX (2 to 100 μM). (F) Graphs showing the mean (± SEM) EFX potentiation of GABA EC_{10}-activated
472 currents mediated by α6β3γ2S and α6β3δ $GABA_A$Rs. Statistical analyses were performed using a non-parametric Mann
473 & Whitney unpaired t-test (* p<0.05; ns: not significant).

In conclusion, taking into account both agonist and potentiating EFX effects, $GABA_AR$s can be ranked in three categories: i) resistant ($\alpha4GABA_AR$s and $\alpha5GABA_AR$s), ii) moderately sensitive ($\alpha1GABA_AR$s, $\alpha2GABA_AR$s and $\alpha6GABA_AR$s) and iii) highly sensitive ($\alpha3GABA_AR$s) to EFX.

3.7 Modelling of EFX-α3β3γ2 $GABA_AR$ interaction

To gain further insight into the mechanism of action of EFX, we generated a homology model of $\alpha3\beta3\gamma2$ $GABA_AR$ (Fig. 7A) to predict how EFX binds to its receptor site. The resulting computed docking model was consistent with an EFX binding site located between α and β subunits in the extracellular domain (Fig. 7B and C). The pocket found at the interface between α3 and β3 subunits, homologous to the GABA binding sites, was large enough to accommodate EFX. Among the putative binding modes, one was found in which EFX bound in the proximity of five amino acid residues of the β3 subunit: N66, Y87, Q89, Y91 and R194 (Fig. 7D). In α3 subunit, we identified two amino acid residues (S257, S258) that may be involved in the EFX-$GABA_AR$ interaction. Two residues of β3, N66 and R194, are conserved in β2 and β3 and are different in β1 subunits (R66 and N194). This pair of amino acids might therefore control the binding mode of EFX as variations at these residues might explain the subunit selectivity.

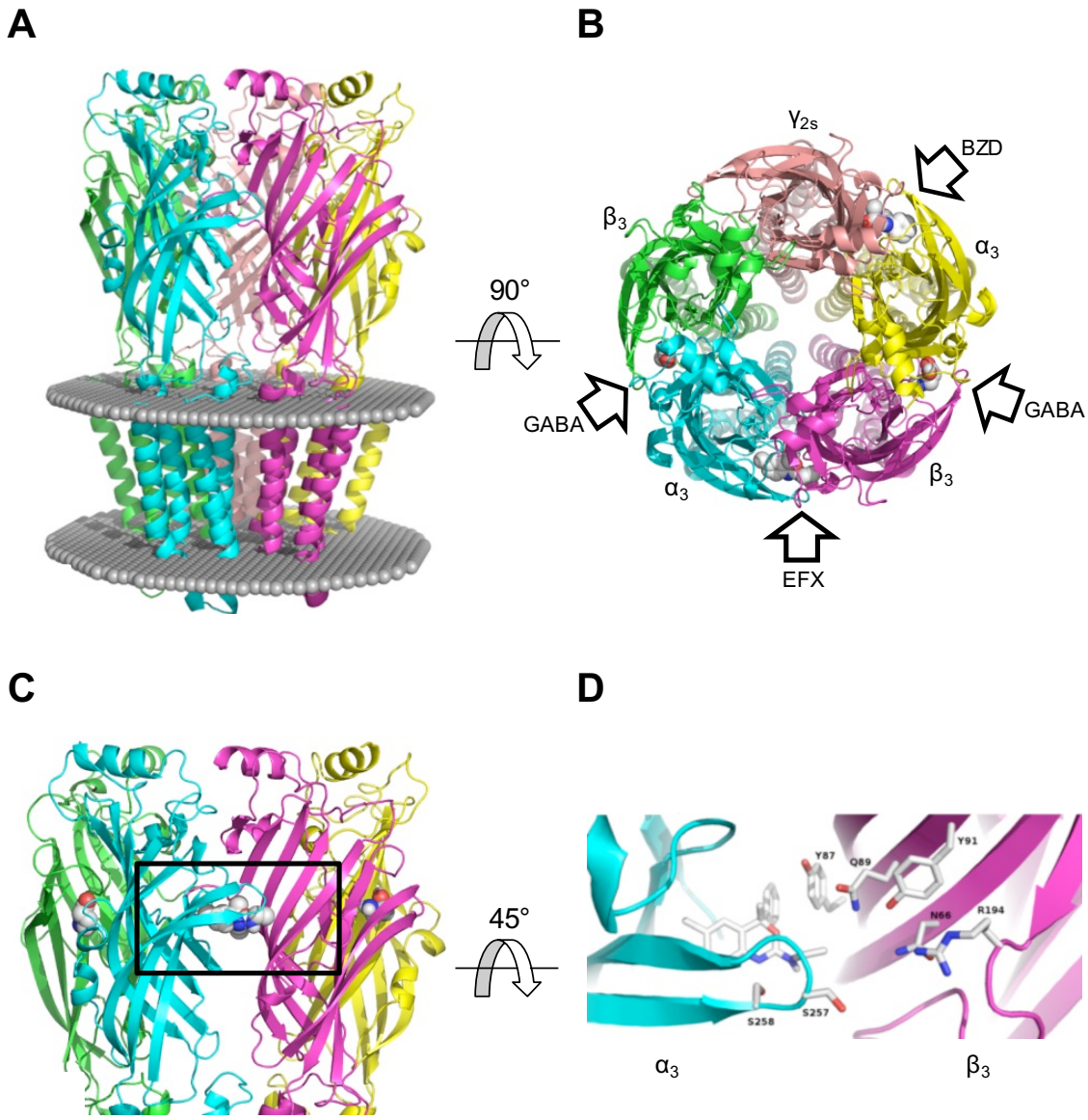

Fig. 7. Binding modes of EFX obtained by docking on the mouse $\alpha_3\beta_3\gamma_{2S}$ $GABA_AR$. (A) Model of the receptor viewed from the membrane plane. The protein is shown in cartoon representation with a different color code for each polypeptide. The position of the membrane is represented by a sphere positioned at the level of lipid head groups as determined by the Orientations of Proteins in Membranes database [51]. (B-D) Binding mode of EFX by docking on the mouse $\alpha_3\beta_3\gamma_{2S}GABA_AR$ model. EFX (CPK representation) interacts with a pocket localized at the α_3 (cyan) β_3 (magenta) interface, homologous to GABA binding sites. The binding sites of BZD, GABA and EFX are indicated by arrows (B). Lateral view of the extracellular domain (C). Close-up showing the EFX-binding pocket (EFX appears in sticks) (D).

4. Discussion

The current study shows that a single administration EFX (25-50 mg/kg) induced a robust anxiolytic behavior in mice subjected to stress-induced hyperthermia and novel object exploration tests. In the same range of doses, and unlike classical BZDs, EFX did not evoke any secondary effects in the spontaneous locomotor activity and rotarod performance. Pharmacokinetic data in Balb/cByJ mice treated with anxiolytic doses indicated that EFX brain content reaches concentration range from 16 to 31 μM [30]. In addition, EFX exhibits lipophilic properties with an estimated partition coefficient (log P) of 2 and a brain/ plasma ratio range of 2.2-2.9 [52]. Based on different reports in the literature, it is reasonable to assume that an equilibrium between the total and free fractions which depends on physicochemical properties of the compound occurs in the brain tissue [53-55] and, as a result this could support the relevance of the effective concentrations in the present electrophysiological studies. In this context, we demonstrated that the α subunit plays an important role in EFX-induced positive effects on $GABA_A$Rs. EFX favours GABA potency over $GABA_A$Rs with the following rank order: α3β3γ2S > α2β3γ2S > α6β3γ2S and no or weak effects on α1β3γ2S, α4β3γ2S and α5β3γ2S.

4.1 EFX displays anxiolytic properties with weak side effects

Our findings confirm the anxiolytic properties of EFX at similar doses to those previously used in other anxiety mouse models [30]. In comparison, EFX displays approximately 50-fold less potent anxiolytic effects than BZP and DZP [8,9]. However, both BZP and DZP strongly alter locomotor performance and awakening at anxiolytic doses, while EFX does not. It is noteworthy that pharmacokinetic factors involving, for example, active metabolites or differences in the extent of metabolism could explain the differences in the effective doses of EFX and BZP. We reasoned that BZDs, which have a high potency (submicromolar) for enhancing GABA-evoked currents, will produce effects at a lower concentration than EFX which has a lower potency (micromolar) for

521 $GABA_AR$s. Interestingly, EFX exhibits higher efficacy for $\alpha2GABA_AR$s and $\alpha3GABA_AR$s, known to
522 mediate anxiolytic effects (up to 171% and 410% for $\alpha2GABA_AR$s and $\alpha3GABA_AR$s, respectively)
523 than DZP (108% and 160% for $\alpha2GABA_AR$s and $\alpha3GABA_AR$s, respectively) [56].

524 Compelling evidence indicates that the anxiolytic effects of BZP cannot be dissociated from
525 its sedative and myorelaxant effects, while the therapeutic margin is wider with EFX. In addition,
526 previous results have shown that EFX is devoid of amnesic effects at anxiolytic doses (50 mg/kg, IP
527 route) in the rat [57]. On the other hand, BZP and DZP display amnesic activity at doses producing
528 anxiolytic effects (from 0.25mg/kg, IP) in the mouse [58]. As observed in rodents, patients treated
529 with EFX for adjustment disorders with anxiety do not exhibit adverse effects, such as the memory
530 and vigilance disturbances [19,59,60]. This is perfectly in line with our electrophysiological data
531 showing the absence of effects of EFX on $\alpha4GABA_AR$s and $\alpha5GABA_AR$s, known to be involved in
532 cognitive functions [4].

533 We cannot rule out the possibility that EFX anxiolytic properties rely on both direct and
534 indirect $GABA_AR$ stimulation mechanisms. Since EFX has been shown to stimulate the synthesis of
535 neurosteroids, such as allopregnenolone, which directly boosts $GABA_AR$ activity [27,43,61], this may
536 account for its anxiolytic effects. Neurosteroids equally enhance GABA-evoked currents mediated
537 by $\alpha1GABA_AR$s, $\alpha3GABA_AR$s and $\alpha6GABA_AR$s, while they have little effects on $\alpha2GABA_AR$s,
538 $\alpha4GABA_AR$s and $\alpha5GABA_AR$s [58,62], suggesting that EFX should induce both sedation and
539 anxiolysis. However, because EFX did not induce sedation at anxiolytic doses, this minors the
540 involvement of neurosteroids in the EFX mode of action. We hypothesize that EFX may exert its
541 anxiolytic effect through a direct enhancement of the activity of $GABA_AR$s. To date, there is no
542 experimental data on EFX modulation of $GABA_AR$s to conclude a plausible mode of action. However,
543 using recombinant murine $GABA_AR$s expressed in *Xenopus* oocytes, it has been shown that both
544 efficacy and potency of GABA are enhanced by EFX [24]. The effect of EFX might be explained by

either mechanism, i.e. an increased frequency of the open state of $GABA_A$Rs and/or an increase of the duration of burst openings.

Further studies using a chronic treatment are warranted to support the specificity of EFX compared to BZDs in the development of tolerance complex phenomenom involving in part selective alterations in $GABA_A$R receptor subunit expression [63].

4.2 The EFX mode of action depends on the $GABA_A$R α subunits

Our electrophysiological data demonstrate that EFX behaves both as a partial agonist and a PAM of $GABA_A$Rs. In fact, EFX strongly potentiates GABA-evoked currents mediated by α2$GABA_A$Rs, α3$GABA_A$Rs and/or α6$GABA_A$Rs, with major effects on α3$GABA_A$Rs in comparison to any other $GABA_A$Rs. This was also highlighted by a larger enhancement of the GABA potency on α3$GABA_A$Rs, than on α1$GABA_A$Rs and α2$GABA_A$Rs. However, the involvement of α3$GABA_A$Rs in anxiolysis is still a matter of debate. As mentioned above, there is still a controversy concerning the implication of α2$GABA_A$Rs, α3$GABA_A$Rs and α5$GABA_A$Rs in the control of anxiety-related behaviors [14,15,17,64]. Other non-BZD compounds such as TPA023, AZD6280 and AZD7325, have been shown to exert anxiolysis without sedative side effects in rodents and/or humans by preferentially enhancing α2$GABA_A$Rs and α3$GABA_A$Rs over the other $GABA_A$R subtypes [64,65]. However, these three compounds bind to the BZD site, while EFX does not [23]. In addition, another non-BZD compound, TP003 was first reported as a selective PAM of α3$GABA_A$R, and initially considered to exhibit anxiolytic properties through this receptor [14,66]. However, two recent studies have revealed that this drug is not selective to α3$GABA_A$R, but equally modulates GABA-evoked currents mediated by α5$GABA_A$R and also moderately potentiates α1$GABA_A$Rs and α2$GABA_A$R [65,67]. TP003 was indeed shown to counteract anxiety behaviors in both rodents and squirrel monkeys and thus highlights the medical use of α3$GABA_A$R-selective molecules as efficient

anxiolytics with no sedative secondary effects [14,66]. Therefore, we believe that the anxiolytic-like effects of EFX in mice are related to the modulation of α2GABA$_A$Rs and α3GABA$_A$Rs.

4.3 The EFX binding site

Our objective was to challenge the possible influence of the α subunit for at least three reasons. First, β$_2$ homopentamers are less sensitive to EFX than β3 homopentamers. However, when they are combined with α1 or α2 subunits, the resulting binary GABA$_A$Rs display a different pharmacological profile: α1β2-3 and α2β2-3 GABA$_A$Rs are equally potentiated, indicating that α1 and α2 subunits modulate EFX potentiation [24]. Second, α1 and α2 subunits share a high amino acid sequence identity, while α4-6 are structurally more distant [68] and thus could potentially have distinct pharmacological influences. Here, we bring compelling evidences demonstrating that stimulating effects of EFX are much stronger on α3β3γ2S than on α2β3γ2S, α6β3γ2S and α1β3γ2S GABA$_A$Rs, while α4β3γ2S and α5β3γ2S are almost insensitive. Altogether, our findings indicate a strong regulatory effect of the α subunit on EFX mode of action.

We also examined the involvement of γ2S and δ subunits in the mode of action of EFX and we observed that α4β3γ2S and α4β3δ on one hand, α6β3γ2S and α6β3δ GABA$_A$Rs on the other hand, are equally sensitive to EFX. This reinforces the idea that, unlike BZDs [7], the third subunit is not involved in the EFX-GABA$_A$R interaction.

Consecutively, we hypothesized that EFX site is likely located between the α and β subunits. Our 3D docking simulation suggests that EFX binds in a pocket at the α/β subunit interface homologous to the GABA binding pocket recently described [69], highlighting putative amino acid residues involved in EFX binding. Interestingly, among them, two residues of β$_3$, N66 and R194, are conserved in β$_2$ and β$_3$ and differ in the β$_1$ subunits (R66 and N194). This pair of amino acids may belong to the binding site of EFX and summarise its subunit selectivity. Site-directed mutagenesis experiments are required to validate this hypothesis and to define the residues underlying the EFX

592 selectivity towards α3GABA$_A$Rs. These experiments will allow us to construct genetic models in
593 which specific α(1-6)GABA$_A$Rs subtypes will be rendered insensitive to EFX to directly correlate
594 specific α2GABA$_A$Rs or α3GABA$_A$Rs to its anxiolytic effects or test whether α3GABA$_A$R functions
595 are involved in the regulation of anxiety.

596 ## 5. Conclusions

597 In conclusion, this study provides new information about the mode of action of EFX, a non-
598 BZD anxiolytic compound, showing that it potentiates GABA transmission, mainly through the
599 interaction with α2GABA$_A$Rs and α3GABA$_A$Rs and likely their associated functions. Modelling
600 simulation indicates that EFX could interact with a pocket localized at the α/β subunits
601 interface,homologous to the GABA binding site. To the best of our knowledge, EFX belongs to the
602 group of non-BZD molecules which act at a site distinct from the classical BZDs site and exert
603 positive effects on anxiety without secondary effects. EFX may therefore serve as a molecular
604 template for the design of novel anxiolytics with similar mechanisms of action and higher potency.

605

606 ## Conflict of interest

607 The authors declare no conflicts of interest.

608

609 ## Acknowledgements

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614

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