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1 **Involvement of the GABA<sub>A</sub> receptor  $\alpha$  subunit in the mode of action of**  
2 **etifoxine**

3  
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## 24 **ABSTRACT**

25 Etifoxine (EFX) is a non-benzodiazepine psychoactive drug which exhibits anxiolytic effects through  
26 a dual mechanism, by directly binding to GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs) and to the mitochondrial 18-  
27 kDa translocator protein, resulting in the potentiation of the GABAergic function. The  $\beta$  subunit  
28 subtype plays a key role in the EFX-GABA<sub>A</sub>R interaction, however this does not explain the anxiolytic  
29 effects of this drug. Here, we combined behavioral and electrophysiological experiments to challenge  
30 the role of the GABA<sub>A</sub>R  $\alpha$  subunit in the EFX mode of action. After single administrations of  
31 anxiolytic doses (25-50 mg/kg, intraperitoneal), EFX did not induce any neurological nor locomotor  
32 impairments, unlike the benzodiazepine bromazepam (0.5-1 mg/kg, intraperitoneal). We established  
33 the EFX pharmacological profile on heteropentameric GABA<sub>A</sub>Rs constructed with  $\alpha$ 1 to  $\alpha$ 6 subunit  
34 expressed in *Xenopus* oocyte. Unlike what is known for benzodiazepines, neither the  $\gamma$  nor  $\delta$  subunits  
35 influenced EFX-mediated potentiation of GABA-evoked currents. EFX acted first as a partial agonist  
36 on  $\alpha$ 2 $\beta$ 3 $\gamma$ 2S,  $\alpha$ 3 $\beta$ 3 $\gamma$ 2S,  $\alpha$ 6 $\beta$ 3 $\gamma$ 2S and  $\alpha$ 6 $\beta$ 3 $\delta$  GABA<sub>A</sub>Rs, but not on  $\alpha$ 1 $\beta$ 3 $\gamma$ 2S,  $\alpha$ 4 $\beta$ 3 $\gamma$ 2S,  $\alpha$ 4 $\beta$ 3 $\delta$  nor  
37  $\alpha$ 5 $\beta$ 3 $\gamma$ 2S GABA<sub>A</sub>Rs. Moreover, EFX exhibited much higher positive allosteric modulation towards  
38  $\alpha$ 2 $\beta$ 3 $\gamma$ 2S,  $\alpha$ 3 $\beta$ 3 $\gamma$ 2S and  $\alpha$ 6 $\beta$ 3 $\gamma$ 2S than for  $\alpha$ 1 $\beta$ 3 $\gamma$ 2S,  $\alpha$ 4 $\beta$ 3 $\gamma$ 2S and  $\alpha$ 5 $\beta$ 3 $\gamma$ 2S GABA<sub>A</sub>Rs. At 20  $\mu$ M,  
39 corresponding to brain concentration at anxiolytic doses, EFX increased GABA potency to the highest  
40 extent for  $\alpha$ 3 $\beta$ 3 $\gamma$ 2S GABA<sub>A</sub>Rs. We built a docking model of EFX on  $\alpha$ 3 $\beta$ 3 $\gamma$ 2S GABA<sub>A</sub>Rs, which is  
41 consistent with a binding site located between  $\alpha$  and  $\beta$  subunits in the extracellular domain. In  
42 conclusion, EFX preferentially potentiates  $\alpha$ 2 $\beta$ 3 $\gamma$ 2S and  $\alpha$ 3 $\beta$ 3 $\gamma$ 2S GABA<sub>A</sub>Rs, which might support  
43 its advantageous anxiolytic/sedative balance.

44

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

47 **Keywords:** etifoxine; GABA<sub>A</sub> receptors;  $\alpha$  subunit; anxiolysis; behavioral pharmacology; EFX-  
48 binding mode

49

50

## 51 **Highlights**

- 52 • We investigated the influence of  $\alpha$  subunits of GABA<sub>A</sub>R on the mode of action of etifoxine,  
53 a non-benzodiazepine compound.
- 54 • Etifoxine induces anxiolysis without locomotion impairment and sedation in mice.
- 55 • Etifoxine strongly potentiates  $\alpha 3\beta 3\gamma 2S$  and moderately  $\alpha 2\beta 3\gamma 2S$  and  $\alpha 6\beta 3\gamma 2S$  GABA<sub>A</sub>Rs  
56 compared to other GABA<sub>A</sub>Rs.
- 57 • A docking model of EFX with  $\alpha 3\beta 3\gamma 2S$  GABA<sub>A</sub>R reveals a binding site at the  $\alpha/\beta$  interface,  
58 close to the GABA-binding pocket.

59

60

61

## 62 **Abbreviations**

63  $\alpha(1-6)$ GABA<sub>A</sub>Rs,  $\alpha 1$  to  $\alpha 6$  subunit-containing GABA<sub>A</sub> receptors; BZD, benzodiazepine; BZP,  
64 bromazepam; DZP, diazepam; EFX, etifoxine; GABA<sub>A</sub>R, GABA<sub>A</sub> receptors; PAM, positive allosteric  
65 modulator; TEVC, two-electrode voltage-clamp

66

## 67 **1. Introduction**

68 GABA<sub>A</sub>Rs are heteropentameric membrane proteins that belong to the cys-loop ligand-gated  
69 ion channel superfamily [1]. They are permeant to chloride ions in response to GABA and decrease  
70 neuronal excitability through membrane hyperpolarization. To date, 19 mammalian GABA<sub>A</sub>R  
71 subunits have been described and cloned ( $\alpha$ 1-6,  $\beta$ 1-3,  $\gamma$ 1-3,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$ ,  $\rho$ 1-3) [1]. The putative  
72 combination of these subunits provides a large heterogeneity of GABA<sub>A</sub>Rs, with a stoichiometry of  
73  $2\alpha$ ,  $2\beta$  and a complementary subunit (mainly  $\gamma$  or  $\delta$ ). The contribution of GABA<sub>A</sub>Rs to fast or slow  
74 neuronal inhibition depends on their stoichiometry, their tissue distribution and their synaptic or  
75 extrasynaptic location. The most frequent assembly of synaptic receptors is  $\alpha(1-3)\beta(1-3)\gamma(1-3)$ ,  
76 whereas extrasynaptic receptors dominantly contain  $\alpha$ 4 or  $\alpha$ 6 with  $\beta(1-3)$  and  $\delta$ , or  $\alpha$ 5,  $\beta(1-3)$  and  
77  $\gamma$ 2 [1,2]. These differences in stoichiometry and distribution support their different  
78 neurophysiological functions and pharmacological properties [3,4].

79 GABA<sub>A</sub>Rs are targeted by benzodiazepines (BZDs) and other drugs for the treatment of  
80 anxiety, epilepsy and sleep disorders [5,6]. BZDs act as positive allosteric modulators (PAMs) of  
81 GABA<sub>A</sub>Rs by binding to a site at the interface between  $\gamma$ 2 subunit and  $\alpha$  subunits [7]. Classical BZDs,  
82 such as diazepam (DZP, Fig. 1), bromazepam (BZP, Fig. 1) and lorazepam, exhibit similar  
83 pharmacological profile in behavioral tests [8,9] and display poor selectivity over GABA<sub>A</sub>Rs which  
84 contain  $\alpha$ 1, or  $\alpha$ 2 or  $\alpha$ 3 or  $\alpha$ 5 ( $\alpha$ 1GABA<sub>A</sub>R,  $\alpha$ 2GABA<sub>A</sub>R,  $\alpha$ 3GABA<sub>A</sub>R, or  $\alpha$ 5GABA<sub>A</sub>R) [10], which  
85 explains their undesirable effects, including withdrawal symptoms, sedation, amnesia, cognitive  
86 impairments and aggressiveness. Indeed,  $\alpha$ 1GABA<sub>A</sub>Rs are associated with sedation, BZD addiction,  
87 anterograde amnesia, anticonvulsant activity and cortical plasticity [10-12].  $\alpha$ 2GABA<sub>A</sub>Rs and  
88  $\alpha$ 3GABA<sub>A</sub>Rs have been linked to anxiolysis, antihyperalgesia and myorelaxation [13-15].  
89  $\alpha$ 5GABA<sub>A</sub>Rs are believed to be correlated to sedation, cognitive impairments and more recently,  
90 anxiolysis [14-17].

91 Etifoxine (2-ethylamino-6-chloro-4-methyl-4-phenyl-4H-3,1-benzoxazine hydrochloride,  
92 EFX, Fig. 1) is a non-BZD compound that exhibits anxiolytic and anticonvulsant effects in rodents  
93 [18] and is used for the treatment of anxiety-related disorders in humans [19,20]. EFX also displays  
94 anti-hyperalgesic and anti-inflammatory properties in different animal models [21,22]. Both *in vitro*  
95 and *in vivo* studies in rats suggested that the anxiolytic effects of EFX involve a dual mechanism of  
96 action, by directly binding to central GABA<sub>A</sub>Rs and to the mitochondrial 18-kDa translocator protein  
97 (TSPO) with, as a result, potentiation of the GABAergic function [23,24]. Indeed, it has been shown  
98 that EFX activates TSPO through a direct binding and consecutively stimulates the synthesis of  
99 neurosteroids, such as, allopregnanolone, which act as PAMs of GABA<sub>A</sub>Rs [25-27]. Although the  
100 affinity of EFX for GABA<sub>A</sub>Rs was twice higher than the one for TSPO (K<sub>d</sub> of 6.1 μM vs K<sub>d</sub> of 12.7  
101 μM), the predominance of one of the effect over the other, i.e. direct GABA<sub>A</sub>Rs binding or through  
102 TSPO activation, in mediating its anxiolytic effect, is still debated [25,28,29].

103 The importance of the β subunit in the mode of action of EFX on GABA<sub>A</sub>Rs has been clearly  
104 evidenced [24]. Constitutively-open homopentameric βGABA<sub>A</sub>Rs are inhibited by EFX. In addition,  
105 α1GABA<sub>A</sub>Rs and α2GABA<sub>A</sub>Rs embedding β2 or β3 are more sensitive to EFX than α1GABA<sub>A</sub>Rs and  
106 α2GABA<sub>A</sub>Rs with β1. These data underline the importance of the β subunit in the EFX-GABA<sub>A</sub>R  
107 interaction and for EFX-potentiation of GABA-induced currents of heteromeric GABA<sub>A</sub>Rs. However,  
108 homopentameric β2GABA<sub>A</sub>Rs are less sensitive to EFX than homopentameric β1GABA<sub>A</sub>Rs [24],  
109 suggesting that the nature of the α subunit might also play a role in EFX-GABA<sub>A</sub>Rs interaction. In  
110 addition, at anxiolytic doses, EFX has no sedative effects nor locomotion impairment in humans [19]  
111 or rodents [30] and this could hardly be explained by the equal potency of EFX on α2GABA<sub>A</sub>R and  
112 α1GABA<sub>A</sub>R since the latter is associated with sedation [10,12]. Thus, we hypothesized that the  
113 pharmacological profile of EFX reflects different sensitivities of all α subtypes containing-GABA<sub>A</sub>Rs.

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

116 and BZP already appear after a single administration [8,9,29,30]. Here, we determined the anxiolytic  
117 doses of EFX and their possible influences on motor performance and arousal. We then assessed the  
118 impact of  $\alpha$  subunit isoforms on the effects of EFX on GABA-evoked currents. We characterized the  
119 pharmacological profile of EFX on murine synaptic GABA<sub>A</sub>Rs ( $\alpha 1\beta 3\gamma 2S$ ,  $\alpha 2\beta 3\gamma 2S$ ,  $\alpha 3\beta 3\gamma 2S$ ,  
120  $\alpha 4\beta 3\gamma 2S$  and  $\alpha 6\beta 3\gamma 2S$ ) and extrasynaptic GABA<sub>A</sub>Rs ( $\alpha 5\beta 3\gamma 2S$ ,  $\alpha 4\beta 3\delta$  and  $\alpha 6\beta 3\delta$ ) using  
121 electrophysiology. This pharmacological study was completed with a 3D model, showing the  
122 interaction between EFX and GABA<sub>A</sub>Rs. Our results demonstrate that the EFX mode of action  
123 involves both  $\alpha$  and  $\beta$ , but not  $\gamma$  or  $\delta$ , subunits.

124

## 125 **2. Materials and methods**

### 126 *2.1 Ethical statements*

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

### 133 *2.2 Animal care and conditioning*

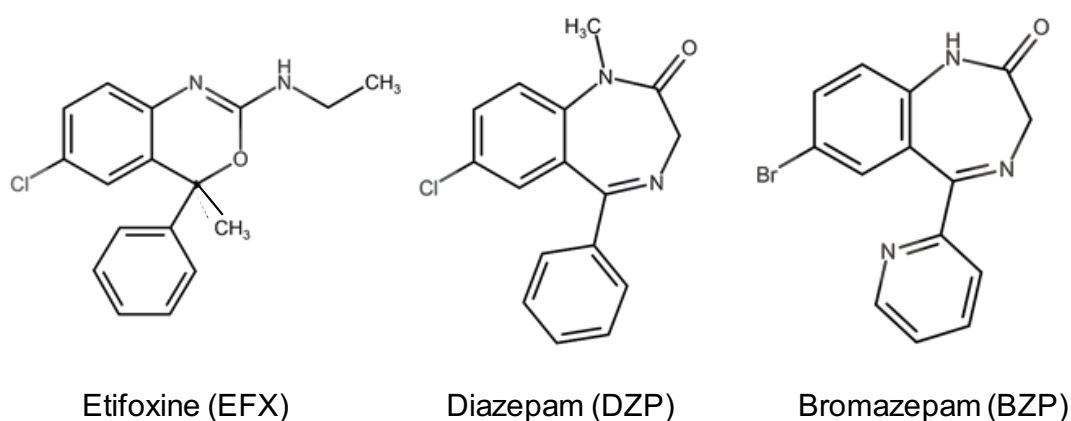
134 Experiments were carried out using 7- to 9-week-old Balb/cByJ mice (25-30 g) purchased  
135 from Charles River Laboratory (Les Oncins, France). Ten mice per translucent polypropylene cage  
136 (internal dimensions in mm: 375 x 375 x 180, L x W x H) were housed under standard laboratory  
137 conditions (22 ± 2° C, 12-h light/dark cycle, lights on at 7:00 AM) with food (AO4, SAFE, France)  
138 and tap water available *ad libitum*. No less than one week of rest followed their arrival. Mice were  
139 habituated to the testing room at least 60 min before performing any behavioral evaluation. All tests  
140 occurred between 9:00 AM and 3:00 PM. The behavioral tests were performed by two well-trained  
141 experimenters, who remained unaware of the administered treatment. In addition, all equipment was  
142 wiped with 70% ethanol between animals to erase the olfactory stimuli. All experiments were  
143 performed in a randomized manner. Single administrations of EFX (12.5-150 mg/kg, expressed as  
144 hydrochloride salt) or BZP (0.25-1 mg/kg) were given by the intraperitoneal (IP) route, 30 min before  
145 each test, except in the stress-induced hyperthermia test in which the compounds were administered  
146 60 min before the test. Studies have shown that both compounds have a similar profile with plasma  
147 peak at 15-30 min [25,31-33]. The control animals received an equivalent volume of vehicle (0.9%  
148 NaCl, 1% tween 80 (v/v)). One male C57Bl/6N mouse was used for the cloning experiments. This



149 mouse had been included in a control group (not treated) from a previous protocol in which mice  
150 were purchased from Janvier Labs (Le Genest-Saint-Isle, France). Euthanasia was performed using  
151 CO<sub>2</sub> (3 ml/min, 4 min). Adult female *Xenopus laevis* were purchased from CRB (Rennes, France) and  
152 had been bred in the laboratory in strict accordance with the recommendations of the Guide for the  
153 Care and Use of Laboratory Animals of the European Community. Oocytes were harvested from  
154 mature female *Xenopus laevis* frogs under 0.15% tricaine anaesthesia. All animals recovered after 2-  
155 3 h. Every female is operated every three months, not less. A single female was used no more than 5  
156 times.

### 157 2.3 Compounds

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



171 **Fig. 1.** Structure of the positive allosteric modulators of GABA<sub>A</sub>Rs used in this study. The 2D structure of EFX (PubChem  
172 CID: 171544), DZP (PubChem CID: 3016) and BZP (PubChem CID: 2441) are illustrated.

173

#### 174 *2.4 Anxiety-related behavioral assessment*

175 Stress-induced hyperthermia (SIH) is defined as the increase in body temperature observed  
176 when a subject is exposed to an external stressor [34]. The day preceding the experiment, the animals  
177 were isolated in smaller cages (dimensions: 265x160x140 mm). The body temperature ( $\pm 0.1^{\circ}\text{C}$ ) of  
178 one singly housed mouse was measured twice via a rectal probe (YSI n°423; 2 mm diameter) coupled  
179 to a thermometer (Letica-Temp812 model-Italia) at an interval of 10 min. The rectal temperature  
180 measurement procedure (handling, insertion of the probe) constitutes the stressor. Drugs were injected  
181 60 min before the first measurement (T1), followed by a second temperature measurement 10 min  
182 later (T2). Methodological experiments have shown that the optimal conditions for drug testing are  
183 found with an injection-test interval of 60 min or longer to avoid residual effects of the injection  
184 procedure. Indeed, using a 60 min-injection-stress interval results in a hyperthermic response  
185 comparable to animals that are not injected [35,36]. The reduction of SIH ( $\Delta T = T2-T1$ ) is considered  
186 to reflect an anxiolytic-like effect [34-36]. Defensive burying after novel object exploration is a  
187 behavior that can be elicited in rodents in response to aversive or new stimuli [37]. Mice were singly  
188 housed in smaller cages (see above) with a sawdust depth of 2.5 cm the day before the test. Each  
189 mouse was confronted with an unfamiliar object (4x4x6 cm; aluminium) introduced in the centre of  
190 the cage. The time of contact or exploring the object (snout pointing toward the object at a distance  
191  $< 1$  cm with or without burying) was recorded for 10 min. The object was cleaned with alcohol (10%)  
192 between each trial. This behavioral approach reveals an anxiety-like or fear state and its suppression  
193 is associated with a reduction of the anxiety-like behavior [37].

#### 194 *2.5 Spontaneous locomotor activity*

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

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

## 202 *2.6 Rotarod performance*

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

## 209 *2.7 GABA<sub>A</sub>R subunit cDNA expression vectors*

210 pRK7 plasmids containing cDNAs encoding rat  $\alpha$ 2 subunits were a kind gift from Professor  
211 Harmut Lüddens (University of Mainz, Mainz, Germany). The GABA<sub>A</sub>R  $\alpha$ 2 subunits from rat and  
212 mouse are identical in amino acid sequence. pGW1 (=pRK5) plasmids containing cDNAs encoding  
213 mouse  $\beta$ 3 and  $\gamma$ 2S subunits were kindly provided by Professor Steven J. Moss (Tufts University,  
214 Boston, USA). The cDNAs encoding the  $\alpha$ 3,  $\alpha$ 4,  $\alpha$ 5,  $\alpha$ 6 and  $\delta$  subunits used in this work were cloned  
215 in mouse brain as described below.

## 216 *2.8 RNA extraction, RT-PCR and cloning of full-length cDNAs encoding $\alpha$ 3-6 and $\delta$ subunits*

217 The brain was dissected from a male C57Bl/6N mouse for RNA extraction and purification.  
218 Total RNA was then extracted using TRIzol® Reagent (Ozyme/Biogentex, France). First strand  
219 cDNAs were synthesised from 5  $\mu$ g of total RNA using SuperScript™ III First-Strand Synthesis  
220 System Super Mix (Invitrogen, USA) in the presence of oligo (dT)<sub>20</sub>, according to the manufacturer's

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

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

## 251 2.9 Expression of GABA<sub>A</sub>Rs in *Xenopus* oocytes

252 Adult female *Xenopus laevis* (CRB, Rennes, France) were anaesthetized in ice-cold water  
253 with 0.15% Tricaine (3-aminobenzoic acid ethyl ester, Sigma). Ovarian lobes were collected and  
254 washed in standard oocyte saline (SOS containing 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM  
255 MgCl<sub>2</sub>, 5 mM HEPES, pH 7.4). Stage V-VI oocytes were partially defolliculated by enzymatic  
256 treatment with 2 mg/ml collagenase (type IA, Sigma) in Ca<sup>2+</sup>-free SOS for 60 min. To express  
257 functional GABA<sub>A</sub>R, cDNA mixtures were directly injected into the nucleus (animal pole) of  
258 individual defolliculated oocytes in different volumes of DNA solution at a concentration of 50 ng/μl  
259 using a nanoinjector (Drummond Nanoject) (see Table S2 for receptor stoichiometry and DNA  
260 quantity injected). Following injection, the oocytes were kept at 18°C in SOS supplemented with  
261 gentamycin (50 μg/ml), penicillin (100 UI/ml), streptomycin (100 μg/ml), and sodium pyruvate  
262 (2.5 mM). The incubation medium was replaced every two days. Oocytes were incubated 1 to 2 days  
263 after DNA injection, depending on the GABA<sub>A</sub>R subtype.

## 264 2.10 Electrophysiological recordings

265 Injected oocytes were tested for GABA<sub>A</sub>R expression, at a holding potential of -60 mV using  
266 a two-electrode voltage clamp amplifier (TEV-200, Dagan Corporation, Minneapolis, USA). Digidata  
267 1440A interface (Axon CNS Molecular Devices, California, USA) and pCLAMP 10 software (Axon  
268 CNS Molecular Devices) were used for acquisition. Cells were continuously superfused with standard  
269 oocyte saline (SOS) at room temperature and were challenged with drugs in SOS. Electrodes were

270 filled with 1 M KCl / 2 M K acetate and had typical resistances of 0.5–2 MΩ in SOS. Drugs were  
271 perfused at a flow rate close to ~4 ml/min. EFX and DZP were applied for 2 min before co-application  
272 of GABA at EC<sub>10</sub>, (determined for each GABA<sub>A</sub>R subtype, see Table S2 in supplementary data) until  
273 the current response peaked. EFX was tested at concentrations ranging from 2 to 100 μM,  
274 corresponding to its clinical use. Between the two applications, the oocytes were washed in SOS for  
275 10-15 min to ensure full recovery from receptor desensitization (see Fig. 4A inbox). To control  
276 whether GABA-evoked currents were mediated by ternary α1-6β3γ2SGABA<sub>A</sub>Rs, control  
277 experiments were performed using SOS containing 10 μM Zn<sup>2+</sup> to inhibit binary GABA<sub>A</sub>Rs [40]. Data  
278 were analysed using pCLAMP 10 software. Data are expressed as the mean ± SEM of 6-10 oocytes  
279 generated from at least two collections. Concentration-effect relationships were analysed using the  
280 following equation:  $Y = Y_{\min} + (Y_{\max} - Y_{\min}) / (1 + 10^{(L \cdot \log(E_{C50} - X) / nH)})$ , where X is the concentration of EFX,  
281 Y<sub>min</sub> and Y<sub>max</sub> are the minimum and highest responses, and nH is the Hill coefficient.

## 282 *2.11 Molecular model design*

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

## 292 *2.12 Docking*

293 The docking had been performed with AutoDock Vina [46]. The ligands and proteins were  
294 prepared with prepare\_ligand4.py and prepare\_receptor4.py scripts, respectively. The side chains of  
295 amino-acids in the binding site were made flexible ( $\alpha$ 3: Ser257 Ser258 and  $\beta$ 3 Gln66, Tyr87, Gln89,  
296 Tyr91). Each ligand was docked 100 times in a large cube of 30 Å in each dimension. Fig. 7 was  
297 prepared with PyMOL (DeLano W.L. (2010) The PyMOL Molecular Graphics System, version 1.6,  
298 Schrodinger, LLC, New York).

### 299 *2.13 Data analysis and statistics*

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

316

### 317 **3. Results**

#### 318 *3.1 Anxiolytic effects of EFX*

319 Previous studies have shown that EFX exhibits anxiolytic effects using conventional  
320 behavioral tests such as elevated plus maze and dark-light box tests [33,47]. Here, we evaluated the  
321 effect of EFX on stress and anxiety-related behaviors (stress-induced hyperthermia and novel object  
322 exploration) to determine and confirm its anxiolytic doses in comparison with BZP. In non-treated  
323 animals, handling stress resulted in a rise in body temperature close to 1°C (Fig. 2A to D). BZP  
324 significantly lowered body temperature in animals at 1 mg/kg dose, before handling (T0) revealing  
325 hypothermia ( $H(3)=11.343$ ,  $p=0.010$ , then  $p<0.05$ , Dunn's test) (Fig. 2A). BZP dose-dependently  
326 prevented stress-induced hyperthermia ( $F_{3,41}=18.290$ ,  $p<0.001$ ) (Fig. 2B). Compared to the vehicle-  
327 treated animals, BZP was effective at doses of 0.5 and 1 mg/kg ( $p<0.05$ , Dunnett's test). EFX also  
328 induced changes in body temperature but without hypothermia at the highest dose (50 mg/kg)  
329 compared with control animals ( $F_{3,41}=2.269$ ,  $p=0.095$ ) (Fig. 2C). As observed for BZP, the  
330 temperature increase was dose-dependently prevented by EFX ( $H(3)=20.072$ ,  $p<0.001$ ) with a  
331 significant effect at 25 and 50 mg/kg dose ( $p<0.05$ , Dunn's test) (Fig. 2D). The anxiolytic effects of  
332 BZP and EFX were also assessed by evaluating the behavioral approach in the presence of an  
333 unfamiliar object. The duration of contact with a novel object was significantly decreased in animals  
334 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).  
335 The same was observed with EFX ( $H(3)=17.536$ ,  $p<0.001$ ), with a significant effect observed at doses  
336 of 25 and 50 mg/kg compared with the control animals ( $p<0.05$ , Dunn's test) (Fig. 2F). In conclusion,  
337 these two different behavioral tests led to similar anxiolytic doses of EFX (25-50 mg/kg, IP).

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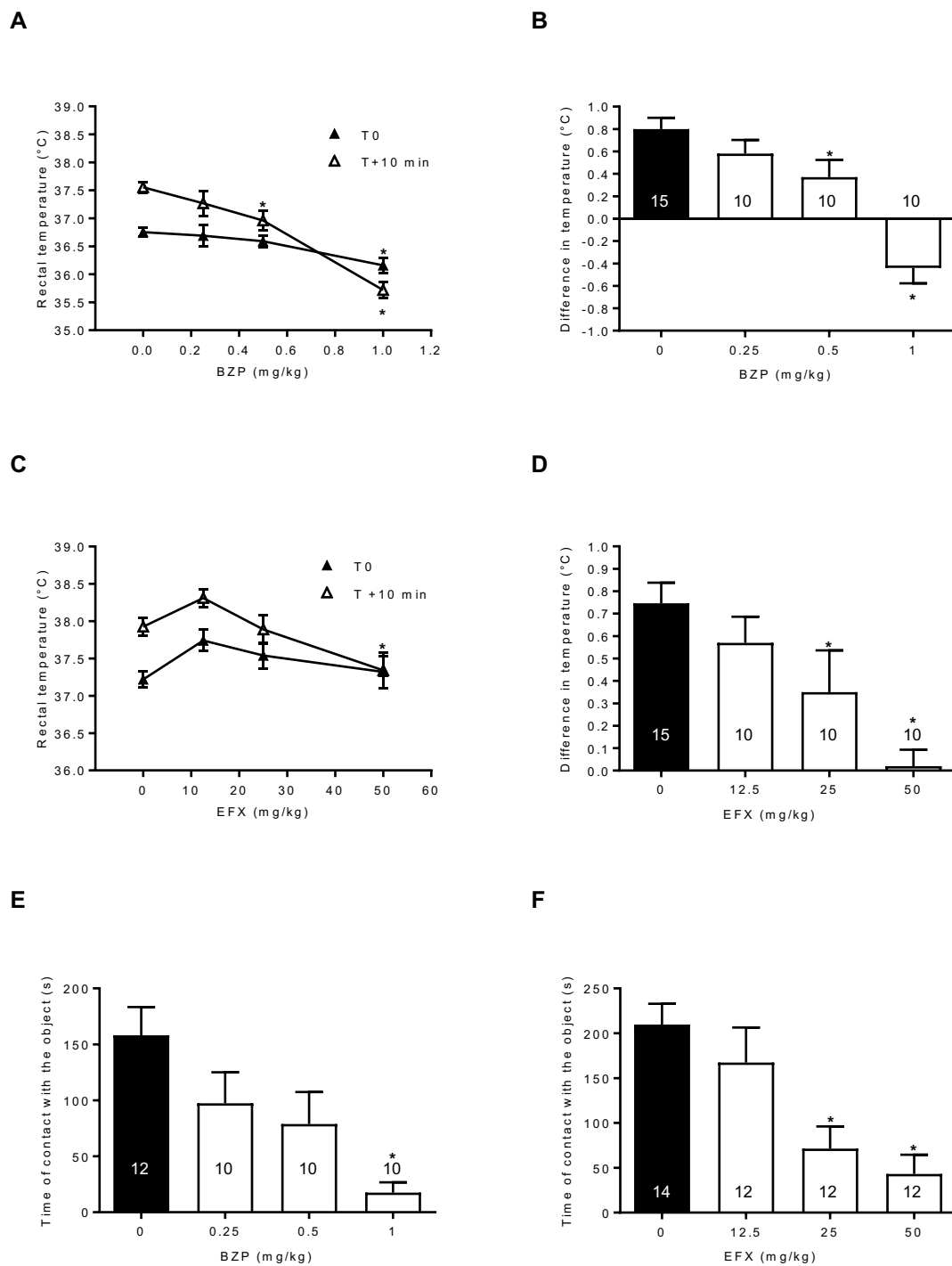
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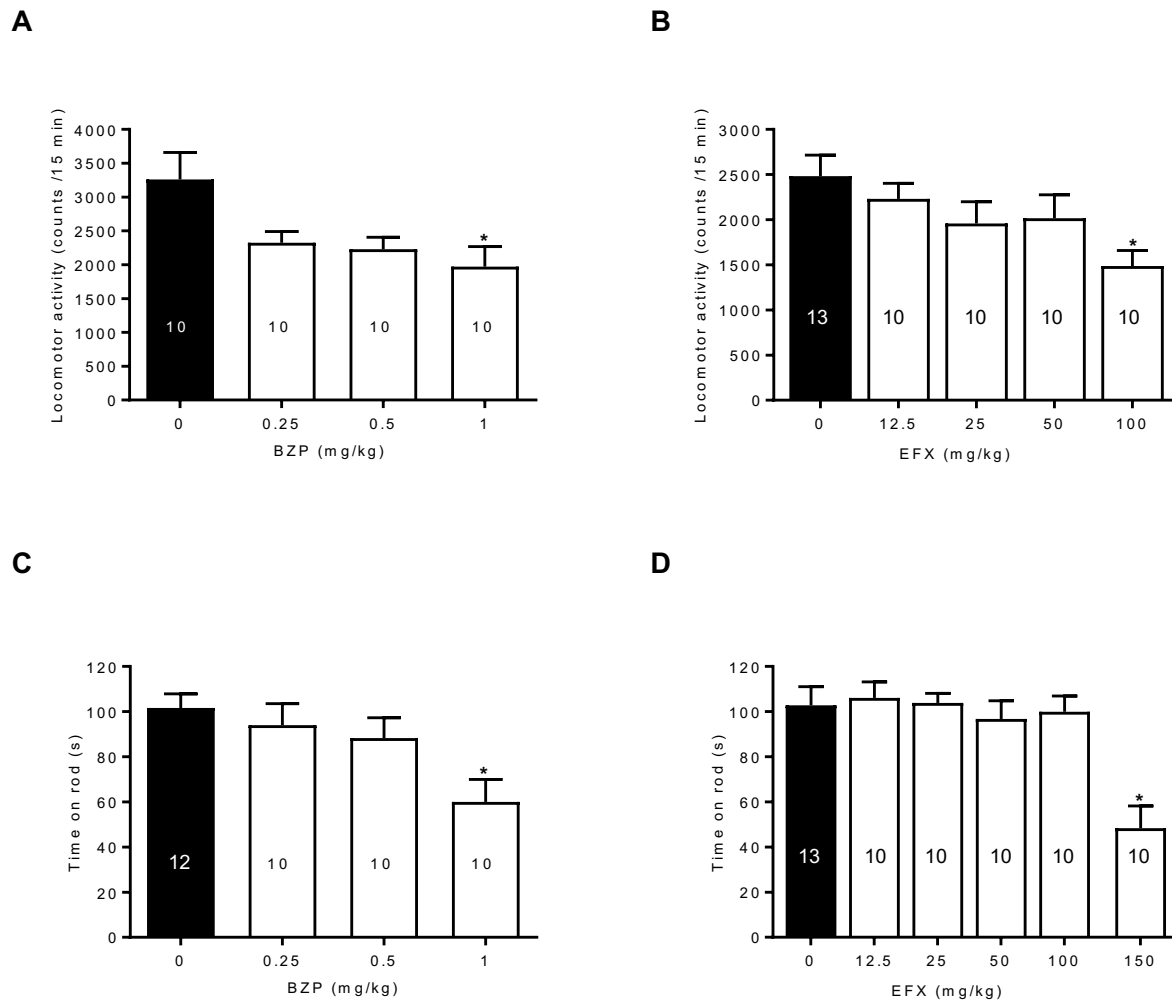
361



362 **Fig. 2.** Comparison of the effects of EFX and BZP on anxiety-related behaviors in mice. (A-D) The graphs show the  
363 evolution of the mean rectal temperature ( $\pm$  SEM) at T0 and T0+10 min after treatment with vehicle (dose 0) or BZP (A)  
364 or EFX (C) through IP route, 60 min before the first temperature measurement at T0. Histograms represent the mean ( $\pm$   
365 SEM) of the difference of the rectal temperature measured at T0 and T+10 min in the same mouse after treatment with  
366 vehicle or BZP (B) or EFX (D) at the indicated doses. \* $p<0.05$  compared with the vehicle group (Dunn test). (E,F)  
367 Histograms illustrate the mean time ( $\pm$  SEM) of contact with an unfamiliar object after IP administration of BZP (E) or  
368 EFX (F). \* $p<0.05$  compared with the respective vehicle groups (dose 0). (see “data analysis and statistics” in the Material  
369 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).



382 **Fig. 3.** Comparison of BZP and EFX on locomotor activity and motor coordination. (A, B) Spontaneous locomotor  
 383 activities were assayed in the actimeter test in mice after IP injections of BZP (A) or EFX (B). The bars represent the  
 384 means  $\pm$  SEM of the number of infrared beam interruptions over 15 min. (C,D) Histograms illustrate the time on the rod  
 385 (mean  $\pm$  SEM) after injection of BZP (C) or EFX (D). Animal numbers are indicated inside the bars. \*p < 0.05 compared  
 386 with the respective vehicle groups (see “data analysis and statistics” in the Material and Methods section).  
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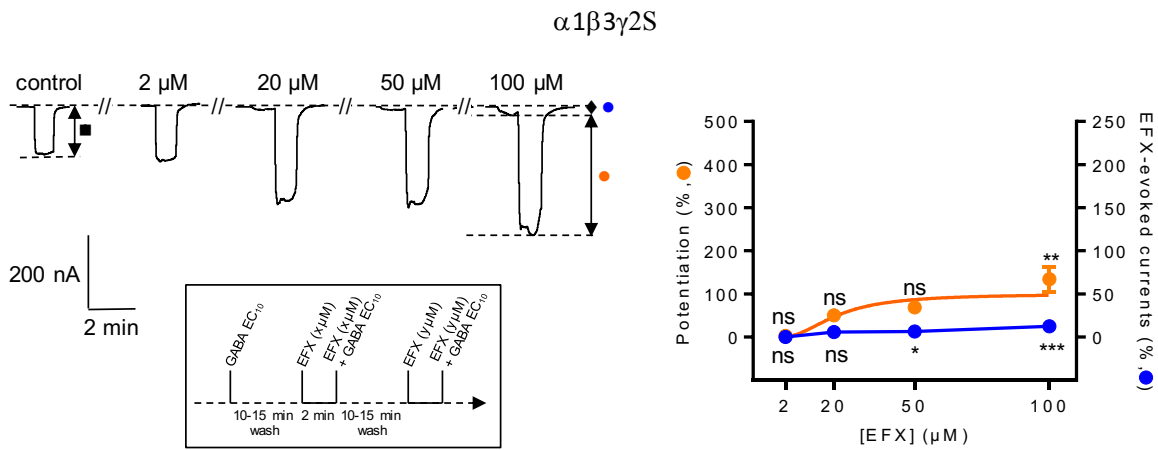
### 388 3.3 EFX effects on GABA currents depends on $\alpha$ subunit isoforms

389 Because the distribution of GABA<sub>A</sub>R  $\alpha$  subunits within the CNS is heterogeneous and  
 390 contributes to their receptor functions, we next investigated the involvement of  $\alpha$  subunits in the EFX  
 391 mode of action using electrophysiology. To achieve this goal, we compared the effects of EFX (2 to  
 392 100  $\mu$ M) on GABA-induced currents elicited by  $\alpha$ 1GABA<sub>A</sub>Rs,  $\alpha$ 2GABA<sub>A</sub>Rs,  $\alpha$ 3GABA<sub>A</sub>Rs,  
 393  $\alpha$ 4GABA<sub>A</sub>Rs,  $\alpha$ 5GABA<sub>A</sub>Rs or  $\alpha$ 6GABA<sub>A</sub>Rs, containing  $\beta$ <sub>3</sub> together with  $\gamma$ 2S or  $\delta$ , when appropriate.

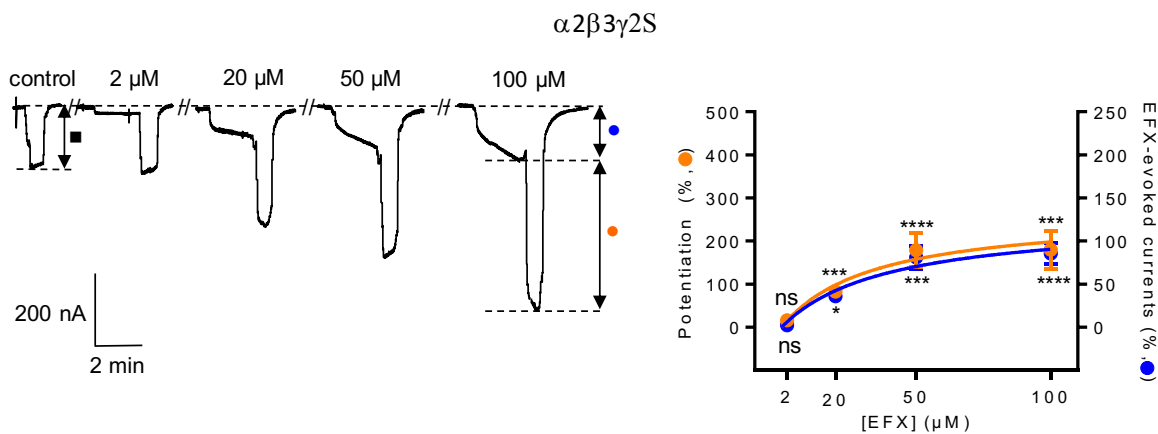
394 We first challenged EFX effects on GABA-currents elicited by synaptic  $\alpha$ 1 $\beta$ 3 $\gamma$ 2S,  $\alpha$ 2 $\beta$ 3 $\gamma$ 2S  
 395 and  $\alpha$ 3 $\beta$ 3 $\gamma$ 2S GABA<sub>A</sub>Rs expressed in *Xenopus* oocytes (Fig. 4). We observed that EFX displays both

396 agonist and potentiating effects as previously described [24]. The agonist effects of EFX were  
397 revealed by its perfusion before co-application with GABA at  $EC_{10}$  (Table S2). EFX exhibits almost  
398 no agonist effects on  $\alpha 1\beta 3\gamma 2S$  GABA<sub>A</sub>Rs ( $12.4 \pm 5.8$  and  $23.7 \pm 10.4$  % of GABA  $EC_{10}$  at 50 and 100  
399  $\mu M$  EFX, respectively) (Fig. 4A). In contrast, comparable agonist effects were observed with  
400  $\alpha 2\beta 3\gamma 2S$  GABA<sub>A</sub>Rs ( $171 \pm 24.9$  % of GABA  $EC_{10}$ , at 100  $\mu M$  EFX, Fig. 4B) and  $\alpha 3\beta 3\gamma 2S$  GABA<sub>A</sub>Rs  
401 ( $153.6 \pm 27.3$ % of GABA  $EC_{10}$ , at 100  $\mu M$  EFX, Fig. 4C). In comparison with GABA, EFX exerted  
402 weaker agonist effects ( $\sim 100$  fold less efficient). For these three receptors, EFX potentiation of GABA  
403  $EC_{10}$ -evoked currents was dose-dependent, reaching a plateau at 50  $\mu M$  (Fig. 4). At this concentration,  
404 EFX induced a potentiation of GABA  $EC_{10}$ -evoked currents by  $68.9 \pm 11.6$ %,  $160.3 \pm 40.2$ % and  
405  $410.7 \pm 20.2$ % of  $\alpha 1\beta 3\gamma 2S$ ,  $\alpha 2\beta 3\gamma 2S$  and  $\alpha 3\beta 3\gamma 2S$ , respectively. The potentiating effects induced  
406 by EFX from 2 to 100  $\mu M$ , was  $\sim 2.4$ - $6.0$ -fold stronger ( $p < 0.05$ ) with  $\alpha 3\beta 3\gamma 2S$  than with  $\alpha 1\beta 3\gamma 2S$   
407 and  $\alpha 2\beta 3\gamma 2S$  GABA<sub>A</sub>Rs. Taking in account both agonist and potentiating effects,  $\alpha 1\beta 3\gamma 2S$  GABA<sub>A</sub>Rs  
408 are found much less sensitive to EFX than  $\alpha 2\beta 3\gamma 2S$  and  $\alpha 3\beta 3\gamma 2S$  GABA<sub>A</sub>Rs.

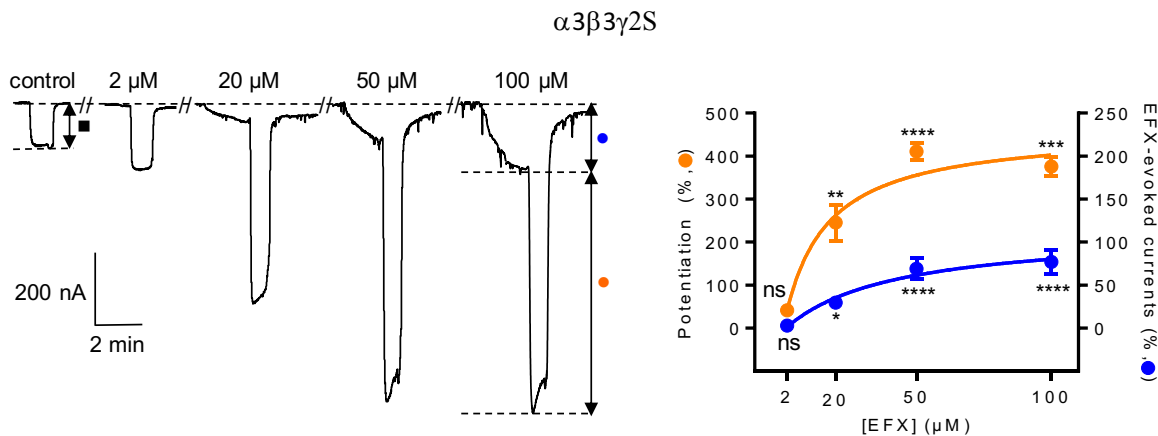
A



B



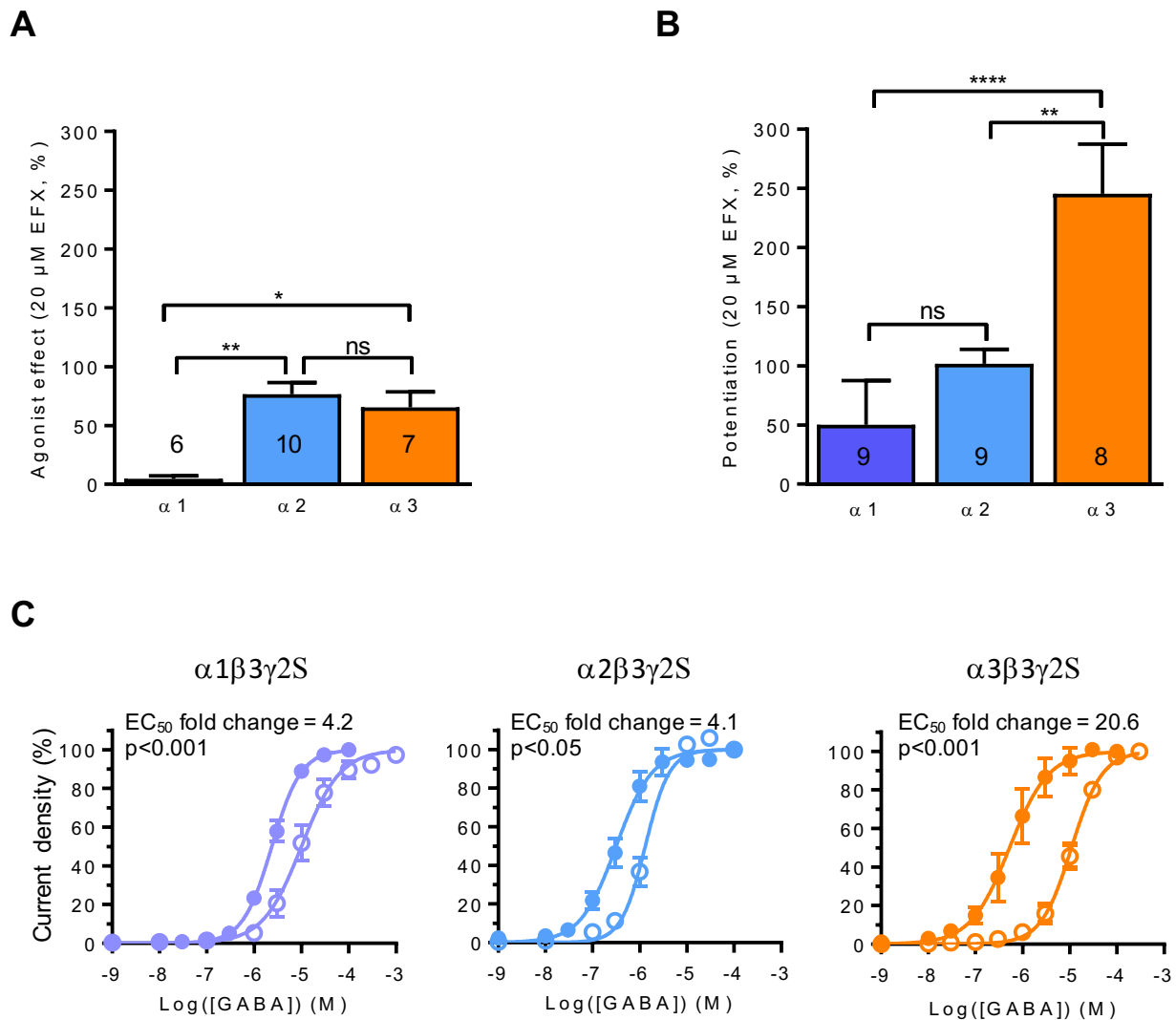
C



409 **Fig. 4.** Effects of EFX on GABA-activated currents mediated by three synaptic GABA<sub>A</sub>Rs ( $\alpha 1\beta 3\gamma 2S$ ,  $\alpha 2\beta 3\gamma 2S$  and  
 410  $\alpha 3\beta 3\gamma 2S$ ). EFX effects were investigated by TEVC in *Xenopus* oocytes expressing  $\alpha 1\beta 3\gamma 2S$  (A),  $\alpha 2\beta 3\gamma 2S$  (B) and  
 411  $\alpha 3\beta 3\gamma 2S$  GABA<sub>A</sub>Rs (C). (A-C) Increasing concentrations of EFX (2, 20, 50 and 100  $\mu M$ ) 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  $\pm$  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  $\mu M$ , \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001; \*\*\*\* $p$ <0.0001, ns: not significant).

419           Based on pharmacokinetic data [30], we estimated that 20  $\mu\text{M}$  matches the concentration of  
420 free EFX in the mouse brain after injection of anxiolytic doses (25-50 mg/kg, Fig. 2). Thus, we  
421 compared agonist and potentiating effects of 20  $\mu\text{M}$  EFX on  $\alpha(1-3)\beta3\gamma2\text{S}$  GABA<sub>A</sub>Rs (Fig. 5A and  
422 B). EFX (20  $\mu\text{M}$ ) acts as a partial agonist on  $\alpha2$ GABA<sub>A</sub>Rs and  $\alpha3$ GABA<sub>A</sub>Rs, while these agonist  
423 effects are not significant on  $\alpha1$ GABA<sub>A</sub>Rs (Fig. 5A). As for the potentiating effects,  $\alpha3$ GABA<sub>A</sub>Rs  
424 were much more sensitive to EFX ( $245.6 \pm 41.8\%$ ) than  $\alpha1$  ( $50.1 \pm 12.5\%$ ) and  $\alpha2$  ( $101.7 \pm 12.2\%$   
425 increase) -containing GABA<sub>A</sub>Rs (Fig. 5B). In the synaptic cleft the GABA concentration rapidly rises  
426 up to the millimolar range [48], we thus compared the GABA concentration-response relationships  
427 in the presence and absence of 20  $\mu\text{M}$  EFX at  $\alpha1$ GABA<sub>A</sub>Rs,  $\alpha2$ GABA<sub>A</sub>Rs and  $\alpha3$ GABA<sub>A</sub>Rs (Fig. 5C,  
428 Table 1). EFX induced a decrease of GABA EC<sub>50</sub> with  $\alpha1$ GABA<sub>A</sub>Rs and  $\alpha2$ GABA<sub>A</sub>Rs in a similar  
429 extent (~4-fold). In contrast, the GABA potency on  $\alpha3$ GABA<sub>A</sub>Rs was increased by 20.6 (Fig. 5C).  
430 Taken together, our electrophysiological data show that, EFX behaves as a selective PAM of  
431  $\alpha3$ GABA<sub>A</sub>Rs at concentration equivalent to anxiolytic doses.

432



433 **Fig. 5.** Pharmacological profile of EFX over  $\alpha(1-3)\beta 3\gamma 2S$  GABA.Rs. Comparison of EFX effects at 20  $\mu$ M corresponding  
 434 to anxiolytic doses. Detailed analysis of agonist (A) and potentiating (B) effects of 20  $\mu$ M EFX on  $\alpha 1\beta 3\gamma 2S$ ,  $\alpha 2\beta 3\gamma 2S$   
 435 and  $\alpha 3\beta 3\gamma 2S$  GABA.Rs. One-way ANOVA followed by Tukey post-hoc test was used for the analysis ( $*p < 0.05$ ,  $**p$   
 436  $< 0.01$ ;  $***p < 0.001$ ;  $****p < 0.0001$ ). The number of recorded oocytes is indicated above or inside the bars. (C)  
 437 Concentration-response curves of GABA-evoked currents in the absence (open circles) and presence (close circles) of 20  
 438  $\mu$ M EFX. Statistical analyses were performed using a non-parametric Mann & Whitney unpaired t-test. All data are  
 439 expressed as the mean  $\pm$  SEM ( $n \geq 6$ ).

440 **Table 1**  
 441 Parameters of the GABA concentration-response relationship at  $\alpha 1\beta 3\gamma 2S$ ,  $\alpha 2\beta 3\gamma 2S$  and  $\alpha 3\beta 3\gamma 2S$   
 442 GABA<sub>A</sub>Rs modulated by 20  $\mu$ M EFX.

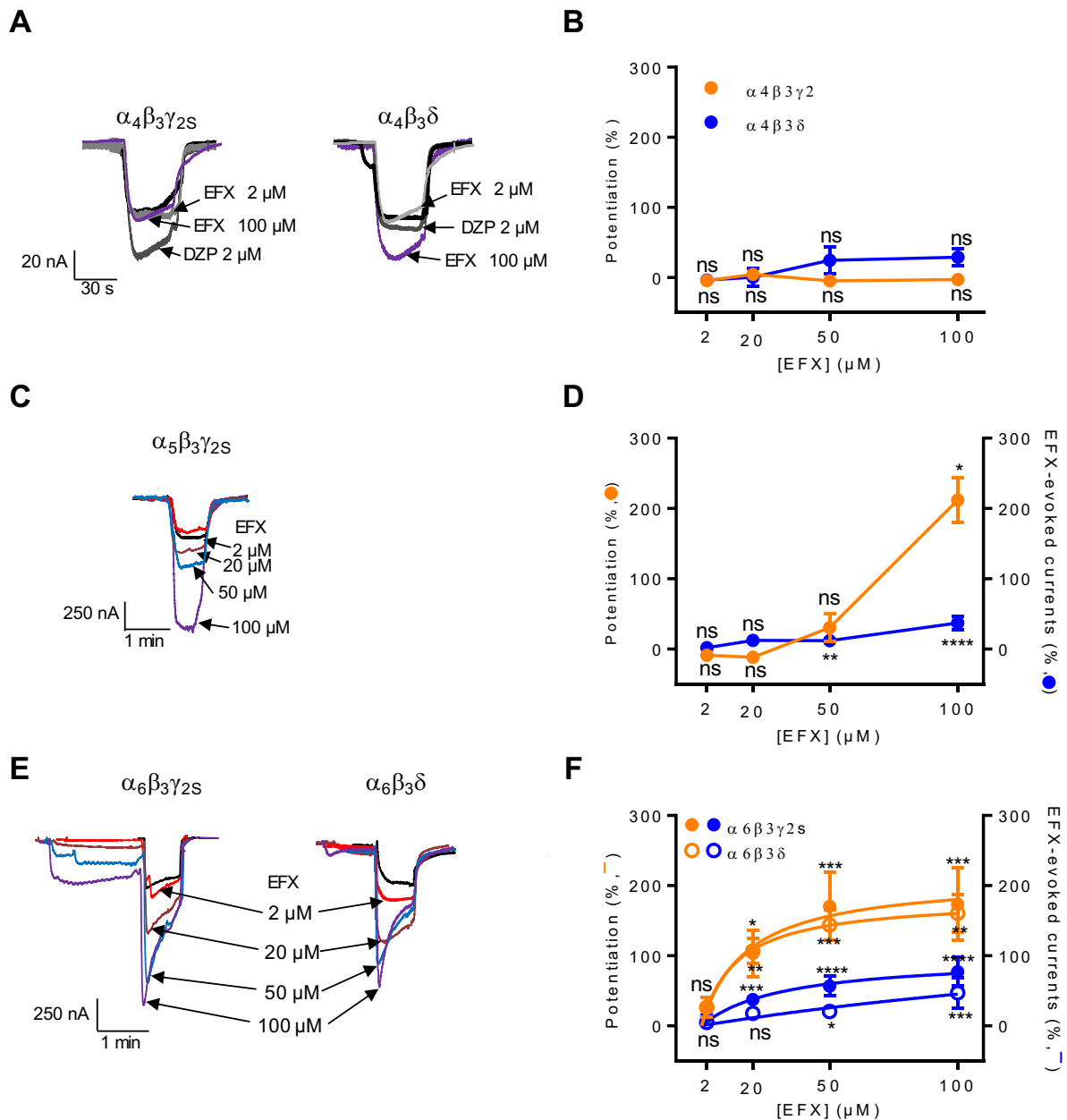
GABA <sub>A</sub> R subtype	control		+ 20 $\mu$ M EFX	
	EC <sub>50</sub> ( $\mu$ M)	nH	EC <sub>50</sub> ( $\mu$ M)	nH
$\alpha 1\beta 3\gamma 2S$	9.99 $\pm$ 0.96	1.06 $\pm$ 0.09	2.37 $\pm$ 0.11	1.41 $\pm$ 0.08
$\alpha 2\beta 3\gamma 2S$	1.31 $\pm$ 0.12	1.69 $\pm$ 0.28	0.32 $\pm$ 0.03	1.14 $\pm$ 0.01
$\alpha 3\beta 3\gamma 2S$	10.89 $\pm$ 0.61	1.34 $\pm$ 0.09	0.53 $\pm$ 0.08	1.05 $\pm$ 0.15

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

445 Next, we tested EFX on synaptic  $\alpha 4\beta 3\gamma 2S$  GABA<sub>A</sub>Rs and extrasynaptic  $\alpha 4\beta 3\delta$  GABA<sub>A</sub>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  $\mu$ M enhanced GABA currents  
448 elicited by  $\alpha 4\beta 3\gamma 2S$  GABA<sub>A</sub>Rs, as previously reported [49], but it did not affect  $\alpha 4\beta 3\delta$  GABA<sub>A</sub>Rs  
449 (Fig. 6A,B). The extrasynaptic  $\alpha 5\beta 3\gamma 2S$  GABA<sub>A</sub>Rs appeared to be also insensitive to EFX at low  
450 concentrations (2 and 20  $\mu$ M), and weakly sensitive to EFX at 50  $\mu$ M ( $54.26 \pm 29.19\%$ ) (Fig. 6C and  
451 D). At 100  $\mu$ M, EFX effects were significantly increased but did not reach a plateau ( $212.0 \pm 31.97\%$ ).  
452 The  $\alpha 6$  subunit was expressed with  $\gamma$  or  $\delta$  in accordance with the native GABA<sub>A</sub>R composition in the  
453 cerebellum [50]. Similar agonist effects were observed with  $\alpha 6\beta 3\gamma 2S$  GABA<sub>A</sub>Rs ( $76.9 \pm 20.3\%$  of  
454 GABA EC<sub>10</sub>, at 100  $\mu$ M EFX,) and  $\alpha 6\beta 3\delta$  GABA<sub>A</sub>Rs ( $46.8\%$  of GABA EC<sub>10</sub>, at 100  $\mu$ M EFX) ( $p=0.23$ ,  
455 Mann and Whitney test) (Fig. 6E,F) as seen with  $\alpha 2$  GABA<sub>A</sub>Rs and  $\alpha 3$  GABA<sub>A</sub>Rs (Fig. 4B,C).  
456 Moreover, the PAM effects of EFX revealed equal sensitivities of synaptic  $\alpha 6\beta 3\gamma 2S$  GABA<sub>A</sub>Rs and  
457 extrasynaptic  $\alpha 6\beta 3\delta$  GABA<sub>A</sub>Rs. EFX-potentiation of GABA-evoked currents reached a plateau at 50  
458  $\mu$ M EFX ( $170.5 \pm 48.4\%$  increase for  $\alpha 6\beta 3\gamma 2S$  GABA<sub>A</sub>Rs and  $143.6 \pm 22.3\%$  for  $\alpha 6\beta 3\delta$  GABA<sub>A</sub>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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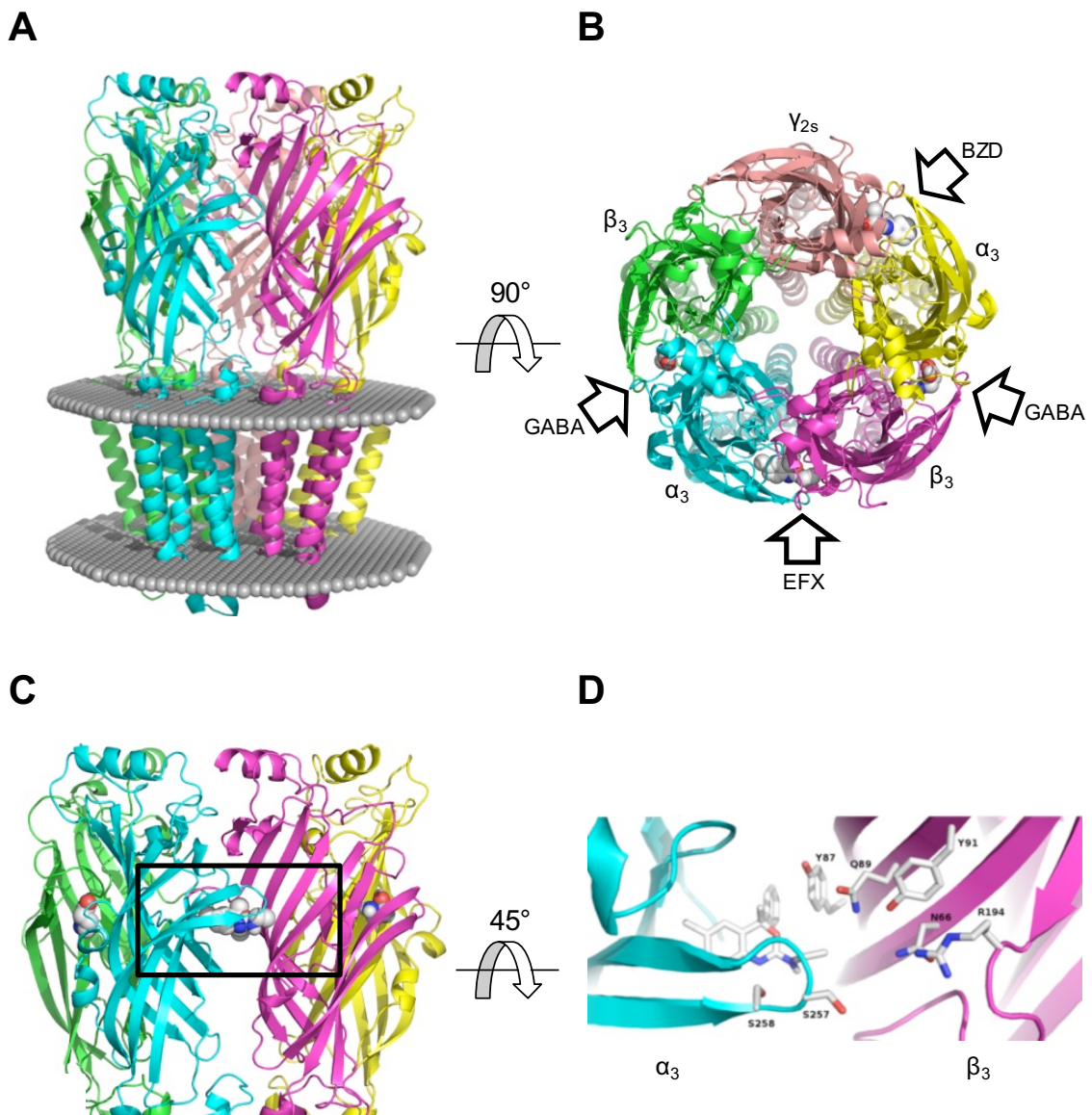
462 **Fig. 6.** Effects of EFX on GABA EC<sub>m</sub>-activated currents mediated by synaptic ( $\alpha_4\beta_3\gamma_2S$  and  $\alpha_6\beta_3\gamma_2S$ ) and extrasynaptic  
 463 ( $\alpha_4\beta_3\delta$ ,  $\alpha_5\beta_3\gamma_2S$  and  $\alpha_6\beta_3\delta$ ) GABA<sub>A</sub>Rs. (A) Superimposed current traces evoked by GABA EC<sub>m</sub> in a representative cell  
 464 expressing  $\alpha_4\beta_3\gamma_2S$  or  $\alpha_4\beta_3\delta$  GABA<sub>A</sub>Rs in the absence (black traces) or presence of DZP (2  $\mu$ M) or EFX. (B) Graphs  
 465 illustrating the mean ( $\pm$  SEM) EFX potentiation of GABA EC<sub>m</sub>-activated currents mediated by synaptic and extrasynaptic  
 466  $\alpha_4$ GABA<sub>A</sub>Rs. (C) Current traces evoked by GABA EC<sub>m</sub> in a representative oocyte expressing extrasynaptic  $\alpha_5\beta_3\gamma_2S$   
 467 GABA<sub>A</sub>Rs in the absence (black trace) or presence of EFX (2 to 100  $\mu$ M). (D) Graphs showing the mean ( $\pm$  SEM) EFX  
 468 potentiation of GABA EC<sub>m</sub>-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<sub>m</sub> with synaptic  $\alpha_6\beta_3\gamma_2S$  and extrasynaptic  $\alpha_6\beta_3\delta$  GABA<sub>A</sub>Rs in the absence (black traces) or presence of increasing  
 471 concentrations of EFX (2 to 100  $\mu$ M). (F) Graphs showing the mean ( $\pm$  SEM) EFX potentiation of GABA EC<sub>m</sub>-activated  
 472 currents mediated by  $\alpha_6\beta_3\gamma_2S$  and  $\alpha_6\beta_3\delta$  GABA<sub>A</sub>Rs. Statistical analyses were performed using a non-parametric Mann  
 473 & Whitney unpaired t-test (\*  $p$ <0.05; ns: not significant).

474 In conclusion, taking into account both agonist and potentiating EFX effects, GABA<sub>A</sub>Rs can  
475 be ranked in three categories: i) resistant ( $\alpha$ 4GABA<sub>A</sub>Rs and  $\alpha$ 5GABA<sub>A</sub>Rs), ii) moderately sensitive  
476 ( $\alpha$ 1GABA<sub>A</sub>Rs,  $\alpha$ 2GABA<sub>A</sub>Rs and  $\alpha$ 6GABA<sub>A</sub>Rs) and iii) highly sensitive ( $\alpha$ 3GABA<sub>A</sub>Rs) to EFX.

### 477 *3.7 Modelling of EFX- $\alpha$ 3 $\beta$ 3 $\gamma$ 2 GABA<sub>A</sub>R interaction*

478 To gain further insight into the mechanism of action of EFX, we generated a homology model  
479 of  $\alpha$ 3 $\beta$ 3 $\gamma$ 2 GABA<sub>A</sub>R (Fig. 7A) to predict how EFX binds to its receptor site. The resulting computed  
480 docking model was consistent with an EFX binding site located between  $\alpha$  and  $\beta$  subunits in the  
481 extracellular domain (Fig. 7B and C). The pocket found at the interface between  $\alpha$ 3 and  $\beta$ 3 subunits,  
482 homologous to the GABA binding sites, was large enough to accommodate EFX. Among the putative  
483 binding modes, one was found in which EFX bound in the proximity of five amino acid residues of  
484 the  $\beta$ 3 subunit: N66, Y87, Q89, Y91 and R194 (Fig. 7D). In  $\alpha$ 3 subunit, we identified two amino acid  
485 residues (S257, S258) that may be involved in the EFX-GABA<sub>A</sub>R interaction. Two residues of  $\beta$ 3,  
486 N66 and R194, are conserved in  $\beta$ 2 and  $\beta$ 3 and are different in  $\beta$ 1 subunits (R66 and N194). This pair  
487 of amino acids might therefore control the binding mode of EFX as variations at these residues might  
488 explain the subunit selectivity.

489



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

## 497 **4. Discussion**

498           The current study shows that a single administration EFX (25-50 mg/kg) induced a robust  
499 anxiolytic behavior in mice subjected to stress-induced hyperthermia and novel object exploration  
500 tests. In the same range of doses, and unlike classical BZDs, EFX did not evoke any secondary effects  
501 in the spontaneous locomotor activity and rotarod performance. Pharmacokinetic data in Balb/cByJ  
502 mice treated with anxiolytic doses indicated that EFX brain content reaches concentration range from  
503 16 to 31  $\mu\text{M}$  [30]. In addition, EFX exhibits lipophilic properties with an estimated partition  
504 coefficient (log P) of 2 and a brain/ plasma ratio range of 2.2-2.9 [52]. Based on different reports in  
505 the literature, it is reasonable to assume that an equilibrium between the total and free fractions which  
506 depends on physicochemical properties of the compound occurs in the brain tissue [53-55] and, as a  
507 result this could support the relevance of the effective concentrations in the present  
508 electrophysiological studies. In this context, we demonstrated that the  $\alpha$  subunit plays an important  
509 role in EFX-induced positive effects on GABA<sub>A</sub>Rs. EFX favours GABA potency over GABA<sub>A</sub>Rs with  
510 the following rank order:  $\alpha 3\beta 3\gamma 2\text{S} > \alpha 2\beta 3\gamma 2\text{S} > \alpha 6\beta 3\gamma 2\text{S}$  and no or weak effects on  $\alpha 1\beta 3\gamma 2\text{S}$ ,  
511  $\alpha 4\beta 3\gamma 2\text{S}$  and  $\alpha 5\beta 3\gamma 2\text{S}$ .

### 512 *4.1 EFX displays anxiolytic properties with weak side effects*

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

521 GABA<sub>A</sub>Rs. Interestingly, EFX exhibits higher efficacy for  $\alpha$ 2GABA<sub>A</sub>Rs and  $\alpha$ 3GABA<sub>A</sub>Rs, known to  
522 mediate anxiolytic effects (up to 171% and 410% for  $\alpha$ 2GABA<sub>A</sub>Rs and  $\alpha$ 3GABA<sub>A</sub>Rs, respectively)  
523 than DZP (108% and 160% for  $\alpha$ 2GABA<sub>A</sub>Rs and  $\alpha$ 3GABA<sub>A</sub>Rs, 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  $\alpha$ 4GABA<sub>A</sub>Rs and  $\alpha$ 5GABA<sub>A</sub>Rs, 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<sub>A</sub>R stimulation mechanisms. Since EFX has been shown to stimulate the synthesis of  
535 neurosteroids, such as allopregnenolone, which directly boosts GABA<sub>A</sub>R activity [27,43,61], this may  
536 account for its anxiolytic effects. Neurosteroids equally enhance GABA-evoked currents mediated  
537 by  $\alpha$ 1GABA<sub>A</sub>Rs,  $\alpha$ 3GABA<sub>A</sub>Rs and  $\alpha$ 6GABA<sub>A</sub>Rs, while they have little effects on  $\alpha$ 2GABA<sub>A</sub>Rs,  
538  $\alpha$ 4GABA<sub>A</sub>Rs and  $\alpha$ 5GABA<sub>A</sub>Rs [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<sub>A</sub>Rs. To date, there is no  
542 experimental data on EFX modulation of GABA<sub>A</sub>Rs to conclude a plausible mode of action. However,  
543 using recombinant murine GABA<sub>A</sub>Rs 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

545 either mechanism, i.e. an increased frequency of the open state of GABA<sub>A</sub>Rs and/or an increase of the  
546 duration of burst openings.

547 Further studies using a chronic treatment are warranted to support the specificity of EFX  
548 compared to BZDs in the development of tolerance complex phenomenon involving in part selective  
549 alterations in GABA<sub>A</sub>R receptor subunit expression [63].

#### 550 *4.2 The EFX mode of action depends on the GABA<sub>A</sub>R $\alpha$ subunits*

551 Our electrophysiological data demonstrate that EFX behaves both as a partial agonist and a  
552 PAM of GABA<sub>A</sub>Rs. In fact, EFX strongly potentiates GABA-evoked currents mediated by  
553  $\alpha$ 2GABA<sub>A</sub>Rs,  $\alpha$ 3GABA<sub>A</sub>Rs and/or  $\alpha$ 6GABA<sub>A</sub>Rs, with major effects on  $\alpha$ 3GABA<sub>A</sub>Rs in comparison to  
554 any other GABA<sub>A</sub>Rs. This was also highlighted by a larger enhancement of the GABA potency on  
555  $\alpha$ 3GABA<sub>A</sub>Rs, than on  $\alpha$ 1GABA<sub>A</sub>Rs and  $\alpha$ 2GABA<sub>A</sub>Rs. However, the involvement of  $\alpha$ 3GABA<sub>A</sub>Rs in  
556 anxiolysis is still a matter of debate. As mentioned above, there is still a controversy concerning the  
557 implication of  $\alpha$ 2GABA<sub>A</sub>Rs,  $\alpha$ 3GABA<sub>A</sub>Rs and  $\alpha$ 5GABA<sub>A</sub>Rs in the control of anxiety-related  
558 behaviors [14,15,17,64]. Other non-BZD compounds such as TPA023, AZD6280 and AZD7325,  
559 have been shown to exert anxiolysis without sedative side effects in rodents and/or humans by  
560 preferentially enhancing  $\alpha$ 2GABA<sub>A</sub>Rs and  $\alpha$ 3GABA<sub>A</sub>Rs over the other GABA<sub>A</sub>R subtypes [64,65].  
561 However, these three compounds bind to the BZD site, while EFX does not [23]. In addition, another  
562 non-BZD compound, TP003 was first reported as a selective PAM of  $\alpha$ 3GABA<sub>A</sub>R, and initially  
563 considered to exhibit anxiolytic properties through this receptor [14,66]. However, two recent studies  
564 have revealed that this drug is not selective to  $\alpha$ 3GABA<sub>A</sub>R, but equally modulates GABA-evoked  
565 currents mediated by  $\alpha$ 5GABA<sub>A</sub>R and also moderately potentiates  $\alpha$ 1GABA<sub>A</sub>Rs and  $\alpha$ 2GABA<sub>A</sub>R  
566 [65,67]. TP003 was indeed shown to counteract anxiety behaviors in both rodents and squirrel  
567 monkeys and thus highlights the medical use of  $\alpha$ 3GABA<sub>A</sub>R-selective molecules as efficient

568 anxiolytics with no sedative secondary effects [14,66]. Therefore, we believe that the anxiolytic-like  
569 effects of EFX in mice are related to the modulation of  $\alpha_2$ GABA<sub>A</sub>Rs and  $\alpha_3$ GABA<sub>A</sub>Rs.

#### 570 *4.3 The EFX binding site*

571 Our objective was to challenge the possible influence of the  $\alpha$  subunit for at least three reasons.  
572 First,  $\beta_2$  homopentamers are less sensitive to EFX than  $\beta_3$  homopentamers. However, when they are  
573 combined with  $\alpha_1$  or  $\alpha_2$  subunits, the resulting binary GABA<sub>A</sub>Rs display a different pharmacological  
574 profile:  $\alpha_1\beta_{2-3}$  and  $\alpha_2\beta_{2-3}$  GABA<sub>A</sub>Rs are equally potentiated, indicating that  $\alpha_1$  and  $\alpha_2$  subunits  
575 modulate EFX potentiation [24]. Second,  $\alpha_1$  and  $\alpha_2$  subunits share a high amino acid sequence  
576 identity, while  $\alpha_{4-6}$  are structurally more distant [68] and thus could potentially have distinct  
577 pharmacological influences. Here, we bring compelling evidences demonstrating that stimulating  
578 effects of EFX are much stronger on  $\alpha_3\beta_3\gamma_2S$  than on  $\alpha_2\beta_3\gamma_2S$ ,  $\alpha_6\beta_3\gamma_2S$  and  $\alpha_1\beta_3\gamma_2S$  GABA<sub>A</sub>Rs,  
579 while  $\alpha_4\beta_3\gamma_2S$  and  $\alpha_5\beta_3\gamma_2S$  are almost insensitive. Altogether, our findings indicate a strong  
580 regulatory effect of the  $\alpha$  subunit on EFX mode of action.

581 We also examined the involvement of  $\gamma_2S$  and  $\delta$  subunits in the mode of action of EFX and  
582 we observed that  $\alpha_4\beta_3\gamma_2S$  and  $\alpha_4\beta_3\delta$  on one hand,  $\alpha_6\beta_3\gamma_2S$  and  $\alpha_6\beta_3\delta$  GABA<sub>A</sub>Rs on the other hand,  
583 are equally sensitive to EFX. This reinforces the idea that, unlike BZDs [7], the third subunit is not  
584 involved in the EFX-GABA<sub>A</sub>R interaction.

585 Consecutively, we hypothesized that EFX site is likely located between the  $\alpha$  and  $\beta$  subunits.  
586 Our 3D docking simulation suggests that EFX binds in a pocket at the  $\alpha/\beta$  subunit interface  
587 homologous to the GABA binding pocket recently described [69], highlighting putative amino acid  
588 residues involved in EFX binding. Interestingly, among them, two residues of  $\beta_1$ , N66 and R194, are  
589 conserved in  $\beta_2$  and  $\beta_3$  and differ in the  $\beta_1$  subunits (R66 and N194). This pair of amino acids may  
590 belong to the binding site of EFX and summarise its subunit selectivity. Site-directed mutagenesis  
591 experiments are required to validate this hypothesis and to define the residues underlying the EFX

592 selectivity towards  $\alpha 3$ GABA<sub>A</sub>Rs. These experiments will allow us to construct genetic models in  
593 which specific  $\alpha(1-6)$ GABA<sub>A</sub>Rs subtypes will be rendered insensitive to EFX to directly correlate  
594 specific  $\alpha 2$ GABA<sub>A</sub>Rs or  $\alpha 3$ GABA<sub>A</sub>Rs to its anxiolytic effects or test whether  $\alpha 3$ GABA<sub>A</sub>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  $\alpha 2$ GABA<sub>A</sub>Rs and  $\alpha 3$ GABA<sub>A</sub>Rs and likely their associated functions. Modelling  
600 simulation indicates that EFX could interact with a pocket localized at the  $\alpha/\beta$  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

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614



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