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# **GABA<sub>A</sub> Receptor Subtype Selectivity of the Proconvulsant Rodenticide TETS**

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## **Abstract**

The rodenticide tetramethylenedisulfotetramine (TETS) is a potent convulsant (lethal dose in humans 7-10 mg) that is listed as a possible threat agent by the United States Department of Homeland Security. TETS has previously been studied *in vivo* for toxicity and *in vitro* in binding assays, with the latter demonstrating it to be a non-competitive antagonist on GABA<sub>A</sub> receptors. In order to determine whether TETS exhibits subtype selectivity for a particular GABA<sub>A</sub> receptor combination, we used whole-cell patch-clamp to determine the potency of TETS on the major synaptic and extrasynaptic GABA<sub>A</sub> receptors associated with convulsant activity. The active component of picrotoxin, picrotoxinin, was used as a control. While picrotoxinin did not differentiate well between 13 GABA<sub>A</sub> receptors, TETS exhibited the highest activity on  $\alpha 2\beta 3\gamma 2$  (IC<sub>50</sub> 480 nM, 95% CI: 320-640 nM) and  $\alpha 6\beta 3\gamma 2$  (IC<sub>50</sub> 400 nM, 95% CI: 290-510 nM). Introducing  $\beta 1$  or  $\beta 2$  subunits into these receptor combinations reduced or abolished TETS sensitivity, suggesting that TETS preferentially affects receptors with  $\alpha 2/\beta 3$  or  $\alpha 6/\beta 3$  composition. Since  $\alpha 2\beta 3\gamma 2$  receptors make up 15-20% of the GABA<sub>A</sub> receptors in the mammalian CNS, we suggest that  $\alpha 2\beta 3\gamma 2$  is probably the most important GABA<sub>A</sub> receptor for the seizure inducing activity of TETS.

## **Keywords**

TETS, GABA<sub>A</sub> receptor, electrophysiology, picrotoxinin, convulsant, threat agent

## Abbreviations

AUC	Area under the curve
CI	Confidence interval
DMSO	Dimethylsulfoxide
EBOB	4'-Ethynyl-4-n-propylbicycloorthobenzoate
EC <sub>50</sub>	Concentration producing 50% effect
E <sub>max</sub>	Maximal effect
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
IC <sub>50</sub>	Concentration producing 50% inhibition
n <sub>H</sub>	Hill coefficient
PTX	Picrotoxinin
SD	Standard deviation
TETS	Tetramethylenedisulfotetramine
TBPS	<i>t</i> -Butylbicyclophosphorothionate

## Introduction

GABA<sub>A</sub> receptors are heteropentameric ligand-gated chloride channels that are activated by gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the adult CNS. GABA<sub>A</sub> receptors have a complex and often somewhat promiscuous pharmacology with numerous orthosteric and allosteric sites that modulate channel function (Krall et al. 2015; Olsen 2015). While GABA<sub>A</sub> receptor agonists and positive allosteric modulators reduce neuronal excitability and can be used as anxiolytics and anticonvulsants, compounds that inhibit GABA<sub>A</sub> receptor functions increase neuronal firing and promote seizures. TETS (tetramethylenedisulfotetramine) and picrotoxin are both potent convulsants (Haskell and Voss 1957; Zolkowska et al. 2012) that can cause severe tonic-clonic seizures and are, therefore, considered threat agents by the United States Department of Homeland Security. Both compounds are thought to work through a similar mechanism; nonselective inhibition of GABA<sub>A</sub> receptors, yet TETS is 30- to 100-fold more potent as a convulsant and lethal toxin in the mouse than picrotoxin (Lamanna and Hart 1968; Shandra et al. 1996).

GABA<sub>A</sub> receptors exhibit a high degree of structural heterogeneity and exist in multiple subtypes (Olsen and Sieghart 2009), with each subtype being a pentamer assembled from a pool of 19 possible subunits,  $\alpha 1$ - $\alpha 6$ ,  $\beta 1$ - $\beta 3$ ,  $\gamma 1$ - $\gamma 3$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\theta$ ,  $\rho 1$ - $\rho 3$ . Based on the fact that TETS and picrotoxin are structurally somewhat similar but distinct (Fig. 1), we wondered if the two agents would exhibit different GABA<sub>A</sub> receptor subtype specificities. Picrotoxin can be isolated from seeds of the moonseed family and has an extremely bitter taste. It is an equimolar mixture of two tricyclic sesquiterpenes; picrotin and the active component picrotoxinin (Slater and Wilson 1951). TETS has similar physicochemical properties, but is easy to synthesize, tasteless and odorless, and stable in drinking water for months (Knaack et al. 2014). These characteristics make TETS a tangible threat.

TETS was first synthesized in 1933 from sulfamide and formaldehyde (Wood and Battye 1933) and then used as an anti-mold agent for upholstery. Its severe toxicity first became apparent in a German furniture factory where workers accidentally exposed to TETS-impregnated wool suffered from

disorientation and seizures (Hagen 1950). TETS was later resynthesized under laboratory conditions and found to be exceptionally poisonous with an extremely low parenteral LD<sub>50</sub> of 0.1-0.3 mg/kg in rodents (Casida et al. 1976; Haskell and Voss 1957). The United States Forestry Department explored TETS as a rodenticide but eventually abandoned it due to its extreme toxicity, the lethal dose in adult humans being 7-10 mg (Guan et al. 1993), the lack of a viable rescue agent, and its persistence in the environment (Whitlow et al. 2005). TETS is currently banned worldwide, but continues to be easily obtainable and popular as a rodenticide in China where several mass poisonings with malicious intent have occurred (Whitlow et al. 2005; Zhang et al. 2011). There also have been cases of reported TETS poisonings in the United States with material sourced from China as an indoor rodenticide (Barrueto et al. 2003; Whitlow et al. 2005). The first pharmacological information about TETS was generated by Alfred Haskell in 1957 (Haskell and Voss 1957), who tested TETS on organ preparations from frogs, rats, cats and dogs and found that TETS acted exclusively on the brain without exhibiting any activity on peripheral nerves or muscle. Seizures only terminated with severance below the medulla. The fact that sub-lethal doses of TETS could reverse pentobarbital-induced decreases in blood pressure and respiration in dogs pointed towards a mechanism potentially involving GABA<sub>A</sub> receptors. TETS was later shown to displace [<sup>35</sup>S]-butylbicyclophosphorothionate ([<sup>35</sup>S]TBPS) binding to rat brain membranes with an IC<sub>50</sub> of 1 μM (Esser et al. 1991; Squires et al. 1983), and to prevent <sup>36</sup>Cl<sup>-</sup> uptake (Ratra et al. 2001) or to inhibit chloride currents (Barnych et al. 2017) through α1β2γ2 GABA<sub>A</sub> receptors expressed in HEK293 cells with IC<sub>50</sub> values of 1.3 or 8 μM, respectively. Interestingly, recent work from the Casida laboratory (Zhao et al. 2014) showed that “cold”-TETS displaces [<sup>14</sup>C]TETS from rat brain membranes with a much lower IC<sub>50</sub> of 80 nM than it displaces another radiolabeled caged convulsant, 4'-ethynyl-4-n-[<sup>3</sup>H]propylbicycloorthobenzoate (EBOB), suggesting that TETS is binding to a site that is only partially overlapping with the EBOB or TBPS site on various GABA<sub>A</sub> receptors or that it might exhibit a specific GABA<sub>A</sub> receptor subtype selectivity. We here tested the later possibility using patch-clamp electrophysiology in an attempt to find an explanation for the discrepancy between the relatively low

potency of TETS in inhibiting GABA<sub>A</sub> receptors and its high *in vivo* toxicity (Lamanna and Hart 1968; Zolkowska et al. 2012).

## Materials and Methods

### Chemicals

Picrotoxinin (PTX), fipronil, bicuculline, propofol, salicylidene salicylhydrazide, zinc chloride, GABA, dexamethasone, zeocin, and geneticin were purchased from Sigma Aldrich (St. Louis, MO, United States). Diazepam, allopregnanolone and DS2 (4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-*a*]pyridin-3-yl]benzamide) were purchased from Tocris Bioscience (Bristol, United Kingdom). TETS was synthesized in the laboratory of Dr. Bruce Hammock, University of California, Davis, CA (Zhao et al. 2014). 10 mM stocks of GABA were made fresh daily using Ringer solution (see below for composition). 10 mM stocks of PTX and TETS were prepared in DMSO and diluted down into Ringer solution immediately before application onto the cell. Both TETS and PTX waste were treated with nitric acid and disposed of using the waste accumulation program at UC Davis.

### Preparation of cells expressing the GABA<sub>A</sub> receptors

The human GABA<sub>A</sub> receptors  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 6$ ,  $\beta 1$ ,  $\beta 3$ ,  $\gamma 2L$ , and  $\delta$  and the rat GABA<sub>A</sub> receptor  $\beta 2$  cloned into pcDNA3.1 expression vectors were a gift from Dr. Robert L. Macdonald, Vanderbilt University, Nashville, TN. The human GABA<sub>A</sub> receptor  $\alpha 4$  cloned into a pcDNA3.1 expression vector was a gift from Dr. Richard Olson, University of California, Los Angeles, CA. The human GABA<sub>A</sub> receptor  $\gamma 1$  cloned into a pcDNA3.1 expression vector and a Lt-K cell line stably expressing  $\alpha 4\beta 3\delta$  (Mortensen et al. 2010) were gifts from Dr. Trevor Smart, University College London, United Kingdom. L929 cells, a mouse fibroblast cell line (CCL-1), were obtained from ATCC (American Type Culture Collection, Manassas, VA, United States), and were used for expressing all GABA<sub>A</sub> receptors with the exception of the receptor combination  $\alpha 6\beta 3\delta$  for which COS-7 cells (ATCC CRL-1651) were used to increase transfection efficacy and expression. L929, Lt-K and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum, 100 U/mL



penicillin and 100 mg/mL streptomycin (Invitrogen, ThermoFisher, Grand Island, NY, United States) and maintained in humidified 95% air and 5% CO<sub>2</sub> air at 37°C. The Lt-K cell line expressing  $\alpha 4\beta 3\delta$  was cultured with 1 mg/mL geneticin and 0.2 mg/mL of zeocin to maintain selection pressure. Two days before experiments, 1  $\mu$ M dexamethasone was added to the media to induce  $\alpha 4$  and  $\beta 3$  expression (Mortensen et al. 2010). L929 or COS-7 cells were transfected using FuGENE 6 (ThermoFisher, Grand Island, NY, United States) transfection reagent in Opti-MEM® reduced serum medium (Life Technologies, Benicia, CA, United States) with an equal amount of each of the subunits (1:1:1) in combination with green fluorescent protein (GFP) expressed from the pEGFP-C1 vector (Invitrogen). The ratio of total cDNA to transfection reagent was 2:1. 48 h post-transfection, cells were detached by trypsinization, washed, plated onto poly-L-lysine coated glass coverslips. Transfected cells were identified as GFP-expressing cells, using an epifluorescence microscope for electrophysiological whole-cell voltage-clamp studies. Correct subunit assembly was tested with a battery of GABA<sub>A</sub> receptor positive allosteric modulators and inhibitors (Table 1).

### **Electrophysiological recordings**

Whole-cell voltage-clamp experiments were performed at room temperature with an EPC-10 HEKA amplifier (HEKA Elektronik, Lambrecht, Germany). Cells were bathed in Ringer solution consisting of 160 mM NaCl, 4.5 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.4, 311 mOsm. Recording electrodes were pulled from soda lime glass micro-haematocrit tubes (Kimble Chase, Rochester, NY, United States) and fire-polished to resistances of 1.8-2.8 M $\Omega$  for voltage-clamp. Electrodes were filled with an internal solution consisting of 154 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES and 10 mM EGTA with pH 7.2 and 305 mOsm. Cells were voltage-clamped at -80 mV and control currents were recorded under the application of varying GABA concentrations for 5 s followed by a 55 s wash with external Ringer solution using a gravity-fed fast perfusion system (VC<sup>3</sup>8 system, ALA Scientific, Farmingdale, NY, United States).

The GABA concentration-response relationships were determined by testing increasing concentrations of GABA and normalizing the GABA induced peak currents to the peak currents induced by a maximal concentration of GABA. The normalized current responses were subsequently fitted using the Hill equation to determine the EC<sub>50</sub> and EC<sub>90</sub>.

For the TETS and PTX concentration-response curves, test solutions were freshly prepared immediately before application onto cells. A near maximal concentration of GABA (GABA EC<sub>90</sub>) was applied for 5 s and then washed out for 55 s. TETS or PTX was then perfused in a volume of 3-5 mL directly into the bath through a side port on the perfusion chamber and cells incubated with the inhibitor for 3 min before a 5 s pulse of GABA was applied to the patch-clamped cell. The convulsant was then washed out and another control GABA pulse applied. One cell was used per concentration of the convulsant. For analysis of receptor blockade, the area under the current curve (AUC<sub>Max</sub>) was determined for the control (EC<sub>90</sub> GABA) and the AUC<sub>Ex</sub> after exposure.

$$\frac{AUC_{Ex}}{AUC_{Max}} \times 100 = \%Blocked$$

For competition experiments, drug was applied for 5 s followed by 55 s of wash with Ringer. The cells were then exposed to GABA or GABA plus the convulsant (TETS or PTX) without any preincubation. GABA-induced responses in the presence and absence of convulsant were quantified by analyzing the peak response.

Data analysis was performed using Excel (Microsoft) and Origin 9.1 (OriginLab Corporation, Northampton, MA, United States) software. Data fitting to the Hill equation to obtain EC<sub>50</sub> or IC<sub>50</sub> values was also performed using Origin 9.1. Individual data points are presented as mean ± SD from 3-8 independent recordings. EC<sub>50</sub> and IC<sub>50</sub> values are presented with 95% confidence intervals.

## Results

### GABA concentration responses

Before studying TETS activity, we first obtained GABA concentration-response curves for thirteen synaptic and extrasynaptic GABA<sub>A</sub> receptor isoforms transiently or stably expressed in L929 cells, a mouse fibroblast cell line that has been used to express GABA<sub>A</sub> receptors since the early 1990s (Angelotti et al. 1993). Correct subunit assembly and  $\gamma$ 2L or  $\delta$  incorporation was confirmed with a battery of GABA<sub>A</sub> receptor probes consisting of the positive allosteric modulators diazepam, propofol, allopregnanolone, and DS2 and the inhibitors fibronil, bicuculline, salicylidene salicylhydrazide and zinc chloride (Table 1). For both the exemplary synaptic GABA<sub>A</sub> receptors  $\alpha$ 1 $\beta$ 1 $\gamma$ 2L,  $\alpha$ 1 $\beta$ 2 $\gamma$ 2L,  $\alpha$ 1 $\beta$ 3 $\gamma$ 2L (Fig. 2A),  $\alpha$ 2 $\beta$ 3,  $\alpha$ 2 $\beta$ 2 $\gamma$ 2L, and  $\alpha$ 2 $\beta$ 3 $\gamma$ 2L (Fig. 2B) and the exemplary extrasynaptic GABA<sub>A</sub> receptors  $\alpha$ 4 $\beta$ 3 $\delta$ ,  $\alpha$ 4 $\beta$ 3 $\gamma$ 2L (Fig. 2C),  $\alpha$ 6 $\beta$ 3 $\delta$ ,  $\alpha$ 6 $\beta$ 3 $\gamma$ 1,  $\alpha$ 6 $\beta$ 2 $\gamma$ 2L,  $\alpha$ 6 $\beta$ 3 $\gamma$ 2L and  $\alpha$ 6 $\beta$ 1 $\gamma$ 2L (Fig. 2D), we determined GABA EC<sub>50</sub> and EC<sub>90</sub> values in order to choose the most appropriate GABA concentrations for subsequently evaluating TETS and PTX effects on each of these receptors. When comparing our EC<sub>50</sub> values to previous work in the field, we found that most of our results are in good agreement with data obtained by Mortson et al. (Mortensen et al. 2011), who transiently expressed various synaptic and extrasynaptic GABA<sub>A</sub> receptors in HEK cells and reported a very similar overall ranking of GABA sensitivity. However, we observed a few differences. In our hands,  $\alpha$ 6 containing combinations were generally 2-3-fold less sensitive to GABA than previously reported. For example, we obtained an EC<sub>50</sub> of 0.69  $\mu$ M for  $\alpha$ 6 $\beta$ 3 $\delta$  (Fig. 2D), whereas Mortenson et al. reported an EC<sub>50</sub> of 0.17  $\mu$ M for this same subunit combination (Mortensen et al. 2011). Despite this difference in sensitivity, we observed the expected pharmacological responses, specifically, no response to diazepam but a pronounced response to DS2 (Table 1).

### Importance of the $\beta$ subunit in TETS activity

Based on molecular dynamics simulations using a homology model of the pore region of the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor, TETS has been hypothesized to bind in the pore lumen by hydrogen bonding with two  $\alpha 1$ -M2 segments and one  $\gamma 2$ -M2 segment without making any contacts with the  $\beta$  subunit (Zhao et al. 2014). In support of this hypothesis, the  $\beta 3$  homopentamer has been shown to not bind TETS even at concentrations greater than 10  $\mu\text{M}$  (Ratra et al. 2001). In order to determine if the  $\beta$  subunit is really not important for TETS activity, as suggested by these studies, we tested all three  $\beta$  subunits,  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ , in combination with  $\alpha 1$  and  $\gamma 2$  (Fig. 3A). We started with  $\alpha 1\beta 2\gamma 2$  since it is the most abundant subunit combination, constituting ~60% of GABA<sub>A</sub> receptors in the mammalian brain (Rudolph and Knoflach 2011; Sur et al. 2001). GABA-induced chloride current at GABA EC<sub>90</sub> through this subunit exhibited a moderate sensitivity to inhibition by TETS (IC<sub>50</sub> 3.6  $\mu\text{M}$ ). Following pre-incubation with different TETS concentrations, block developed quickly, and TETS inhibition was fully reversible on washout; however, even concentrations as high as 100  $\mu\text{M}$  could not fully block the current and only achieved a maximal inhibition of ~70%. The remaining GABA-induced chloride current could be blocked by 100  $\mu\text{M}$  of bicuculline or 10  $\mu\text{M}$  of fipronil (data not shown). To determine the significance of changing the  $\beta$  subunit, we replaced it with both  $\beta 1$  and  $\beta 3$ . While  $\alpha 1\beta 1\gamma 2$  was not very sensitive to TETS (maximal block <50% at 100  $\mu\text{M}$ ),  $\alpha 1\beta 3\gamma 2$  was indistinguishable from the  $\beta 2$  containing subunit combination in terms of TETS potency and maximal efficacy (Fig. 3A). We, therefore, concluded that both  $\beta 2$  and  $\beta 3$  subunits can modulate TETS binding.

### **Understanding the significance of the $\alpha$ subunit in TETS activity on GABA<sub>A</sub> receptors**

We next investigated the role of the  $\alpha$  subunit in TETS activity by exchanging  $\alpha 1$  for  $\alpha 2$ ,  $\alpha 4$  and  $\alpha 6$  in the  $\alpha X\beta 3\gamma 2\text{L}$  combination (Fig. 3B). We chose this combination rather than the  $\alpha X\beta 2\gamma 2\text{L}$  combination because the  $\beta 3$  we are using for this work is human whereas the  $\beta 2$  is a rat clone. We did not investigate  $\alpha 5$  because this subunit is not thought to be involved in seizure generation but rather plays a role in learning and memory (Collinson et al. 2002; Rudolph and Mohler 2004). Out of the tested subunit

combinations,  $\alpha 2\beta 3\gamma 2L$  ( $IC_{50} = 480$  nM with a maximal block of  $\sim 80\%$ ) and  $\alpha 6\beta 3\gamma 2L$  ( $IC_{50} = 400$  nM with a maximal block of  $\sim 75\%$ ) showed the highest sensitivity to TETS (Fig. 3B), while  $\alpha 1\beta 3\gamma 2L$  was roughly 10-fold less sensitive ( $\alpha 1\beta 3\gamma 2L$ :  $IC_{50} = 3.6$   $\mu M$  with a maximal block of  $\sim 75\%$ ). On the  $\alpha 4\beta 3\gamma 2L$  combination, even 100  $\mu M$  of TETS could not achieve more than 45% of inhibition, making this subunit combination the least sensitive.

### **Does the $\beta$ subunit affect the TETS sensitivity of $\alpha 2$ and $\alpha 6$ ?**

We had seen that there was no difference in TETS sensitivity between  $\beta 2$  and  $\beta 3$  when these subunits were expressed with  $\alpha 1$  (Fig. 3A). It was important to determine if this was also true for the more sensitive  $\alpha 2$  and  $\alpha 6$  subunits and we therefore next co-expressed these two subunits with  $\beta 2$  instead of  $\beta 3$ . In both combinations, TETS clearly showed a preference for  $\beta 3$  containing subunit combinations over  $\beta 2$  containing combinations (Fig. 4A and 4B). For the  $\alpha 2\beta X\gamma 2L$  combination, replacing  $\beta 3$  with  $\beta 2$  shifted the TETS concentration-response curve significantly to the right, and increased the  $IC_{50}$  from 400 nM to 12.2  $\mu M$  (Fig. 4A). For the  $\alpha 6\beta X\gamma 2L$  combination, the effect was even more dramatic, and in the presence of  $\beta 2$ , even 100  $\mu M$  of TETS no longer achieved more than 40% inhibition of GABA-induced chloride current (Fig. 4B). Replacing  $\beta 3$  with  $\beta 1$  further reduced TETS activity with 100  $\mu M$  of TETS only achieving 25% inhibition of current through  $\alpha 6\beta 1\gamma 2L$  (Fig. 4B). Based on these results, we conclude that the presence of an  $\alpha 2$  or  $\alpha 6$  subunit and a  $\beta 3$  subunit is necessary for TETS to inhibit chloride currents through GABA<sub>A</sub> receptors with high potency and efficacy.

### **Significance of the $\gamma$ and $\delta$ subunits in TETS activity**

We next tested if  $\delta$  could possibly play a role in TETS activity, and therefore determined its potency for blocking currents through the extrasynaptic  $\alpha 4\beta 3\delta$  GABA<sub>A</sub> receptor stably expressed in Lt-K cells and the  $\alpha 6\beta 3\delta$  receptor transiently transfected into COS-7 cells. We used COS-7 cells for these experiments because the  $\alpha 6\beta 3\delta$  combination did not express well in L929 cells (peak currents  $< 200$  pA). We chose

not to try and generate the  $\alpha 2\beta 3\delta$  GABA<sub>A</sub> receptor since there seems to be no evidence for the existence of this subunit combination in literature (Olsen and Sieghart 2008; Olsen and Sieghart 2009). Interestingly, in the two  $\delta$  containing subunit combinations we investigated, the presence of a  $\delta$  subunit increased the maximal efficacy of TETS inhibition when compared with the  $\gamma 2L$  containing GABA<sub>A</sub> receptor combinations, and also changed the Hill coefficient (Fig. 4C and 4D). But it should be noted here that the two  $\delta$  containing subunit combinations in our study,  $\alpha 4\beta 3\delta$  and  $\alpha 6\beta 3\delta$ , were expressed in two different cell lines, Lt-K and COS-7, which could have impacted our results.

Overall,  $\alpha 4$  containing receptors are not very sensitive to TETS ( $\alpha 4\beta 3\gamma 2L$ :  $IC_{50} = 1.82 \mu M$ ,  $E_{max} = 46\%$ ,  $n_H = 0.7$ ) as previously shown in Fig. 3, but TETS inhibited the  $\delta$  containing  $\alpha 4\beta 3\delta$  receptor with an increased efficacy ( $IC_{50} = 2.66 \mu M$ ,  $E_{max} = 85\%$ ; Fig. 4C) and a steeper Hill coefficient ( $n_H = 1.2$ ). The same effect was observed in the more TETS-sensitive  $\alpha 6$  combination (Fig. 4D). While  $\alpha 6\beta 3\delta$  was less sensitive ( $IC_{50} = 3.69 \mu M$ ) to TETS inhibition than the  $\alpha 6\beta 3\gamma 2L$  receptor ( $IC_{50} = 400 \text{ nM}$ ,  $E_{max} = 64\%$ ,  $n_H = 0.7$ ), TETS displayed a greater efficacy ( $E_{max} = 85\%$ ) and a steeper Hill coefficient ( $n_H = 1.3$ ) for  $\alpha 6\beta 3\delta$  (Fig. 4D). In contrast, exchanging  $\gamma 2$  for  $\gamma 1$  “simply” shifted the TETS concentration-response curve to the right and reduced potency but maintained efficacy ( $\alpha 6\beta 3\gamma 1$ :  $IC_{50} = 6.81 \mu M$ ,  $E_{max} = 60\%$ ). We further tested  $\alpha 2\beta 3$  to see how removing the  $\gamma$  subunit would change TETS sensitivity and found that the absence of a  $\gamma$  subunit in the receptor pentamer lowered both the potency and efficacy of TETS (Table 2).

### **Picrotoxinin shows no GABA<sub>A</sub> receptor subtype selectivity**

As a control, we tested picrotoxinin (PTX), which is a widely used GABA<sub>A</sub> receptor inhibitor also classified as a threat agent on the same receptor subtypes (Table 2 and Fig. 5). As expected based on data in the published literature, PTX is a relatively nonselective GABA<sub>A</sub> receptor inhibitor that blocks GABA induced Cl<sup>-</sup> currents through most GABA<sub>A</sub> receptors with  $IC_{50}$ s in 2-8  $\mu M$  range and an  $E_{max}$  of ~80% current inhibition. Similar to TETS, the presence of a  $\beta 1$  subunit abolishes PTX sensitivity, since

$\alpha 1\beta 1\gamma 2L$  is insensitive to PTX, in contrast to various other  $\beta 2$  (Fig. 5A) or  $\beta 3$  (Fig. 5B) containing GABA<sub>A</sub> receptor combinations, which are all inhibited by PTX (Table 2).

### **TETS is a non-competitive GABA<sub>A</sub> receptor inhibitor**

Lastly, in order to start probing the mechanism of action of TETS on the  $\alpha 2\beta 3\gamma 2L$  receptor, we tested the effect of 2.5  $\mu M$  TETS, which corresponds to the IC<sub>80</sub> on this receptor subtype, on the GABA concentration-response curve (Fig. 5C). As expected from the previously performed binding experiments (Zhao et al. 2014), TETS depressed the maximum GABA response elicited at 50 or 100  $\mu M$  GABA and could not be competed off when the GABA concentration was increased to 1 mM demonstrating that TETS is a non-competitive inhibitor similar to PTX, which was also tested at IC<sub>80</sub> for comparison. PTX at 10  $\mu M$  produced a similar depression of the GABA E<sub>max</sub> and was also not able to shift the GABA concentration-response curve fully to the right (Fig. 5C). Similar results were obtained with the other highly TETS-sensitive GABA receptor combination,  $\alpha 6\beta 3\gamma 2L$  (data not shown).

## Discussion

The rodenticide TETS has previously been primarily studied in animal models to assess its convulsant activity and toxicity or in binding assays, which demonstrated that TETS acts as a non-competitive GABA<sub>A</sub> receptor inhibitor. However, so far only very limited electrophysiological information is available for this highly toxic threat agent. In 1975, Bowery *et al.* demonstrated that TETS could reverse the effects of GABA on isolated superior cervical rat ganglions at concentrations between 10 and 100  $\mu$ M (Bowery *et al.* 1975), while a group in the former Soviet Union showed in 1989 that 5  $\mu$ M of TETS increased the excitability of hippocampal slices (Pervukhin *et al.* 1989). A more recent paper from our own group describing the synthesis of TETS related haptens for the development of an ELISA assay to detect TETS (Vasylieva *et al.* 2017) reported that TETS inhibited chloride currents through  $\alpha$ 1 $\beta$ 2 $\gamma$ 2 GABA<sub>A</sub> receptors expressed in HEK293 cells with an IC<sub>50</sub> of 8  $\mu$ M (Barnych *et al.* 2017). Since we were somewhat surprised by this relatively low potency in electrophysiological assays considering TETS has a reported LD<sub>50</sub> of 0.1 mg/kg (Casida *et al.* 1976; Haskell and Voss 1957), we here investigated the GABA<sub>A</sub> receptor subtype selectivity of TETS using whole-cell patch-clamp electrophysiology. One observation that made us suspect that TETS might exhibit subtype selectivity was a report from the Casida laboratory that unlabeled TETS displaced [<sup>14</sup>C]TETS from rat brain membranes with an IC<sub>50</sub> of 80 nM (Zhao *et al.* 2014), which is much lower than the IC<sub>50</sub>s usually reported for TETS displacement of other radiolabeled GABA<sub>A</sub> receptor antagonists such as EBOB (Zhao *et al.* 2014) or TBPS (Esser *et al.* 1991; Squires *et al.* 1983). Another report suggesting the possibility of a higher affinity target were Ca<sup>2+</sup> dynamics measurements in mouse hippocampal neuronal cultures which develop spontaneous network activity after about 2 weeks in culture (Cao *et al.* 2012). TETS visibly altered Ca<sup>2+</sup> dynamics in these networks at submicromolar concentrations, although the reported EC<sub>50</sub>s for altering the frequency and amplitude of the Ca<sup>2+</sup> induced fluorescence changes in the assay were the 1-2  $\mu$ M range (Cao *et al.* 2012).

Here, we identified two GABA<sub>A</sub> receptor subtypes that are sensitive to TETS at submicromolar concentrations;  $\alpha$ 2 $\beta$ 3 $\gamma$ 2 with an IC<sub>50</sub> of 480 nM and  $\alpha$ 6 $\beta$ 3 $\gamma$ 2 with an IC<sub>50</sub> of 400 nM. Of these two receptor



subtypes, both of which are among the 11 GABA<sub>A</sub> receptors conclusively identified as native receptors (Olsen and Sieghart 2008),  $\alpha 2\beta 3\gamma 2$  is probably the more important receptor for the seizure-inducing activity of TETS since  $\alpha 2\beta 3\gamma 2$  receptors make up 15-20% of the GABA<sub>A</sub> receptors in the mammalian CNS (Fritschy and Mohler 1995; Pirker et al. 2000; Rudolph and Knoflach 2011) and  $\alpha 2$  containing GABA<sub>A</sub> receptors have been shown to significantly contribute to the anticonvulsant actions of diazepam (Fradley et al. 2007). In contrast, receptors with the  $\alpha 6\beta 2/3\gamma 2$  composition constitute less than 5% of the GABA<sub>A</sub> receptors (Mohler et al. 2002; Rudolph and Knoflach 2011) and are largely restricted to the cerebellum (Jones et al. 1997). One short coming of our study is that we only used the long splice variant of the  $\gamma 2$  subunit,  $\gamma 2L$ , and not the short  $\gamma 2s$  subunit, which is known to be expressed throughout the mammalian CNS in similar proportions and often co-localizes with  $\gamma 2L$  in the same receptor complexes (Khan et al. 1994).

TETS differs from the less selective PTX in showing preference for  $\alpha 2$  and  $\alpha 6$  over  $\alpha 1$  and  $\alpha 4$  when expressed in combination with  $\beta 3$  and  $\gamma 2$ . Introducing a  $\beta 1$  subunit into the GABA<sub>A</sub> receptor heteropentamer dramatically decreases both TETS and PTX potency and efficacy, suggesting that the presence of an  $\alpha/\beta 1$  interface disrupts binding for both compounds. In contrast, exchanging  $\beta 3$  for  $\beta 2$  or  $\gamma 2$  for the less commonly found  $\gamma 1$  reduces TETS activity by roughly 10-fold (Table 2). However,  $\beta 3$  alone is not sufficient to generate a TETS binding site since homopentameric  $\beta 3$  GABA<sub>A</sub> receptors have been shown to not bind TETS at concentrations of up to 10  $\mu\text{M}$  while they bind PTX with an affinity of 32 nM (Ratra et al. 2001). It is interesting that the presence of a  $\beta 3$  subunit in the preferred  $\alpha 2/\beta 3$  or  $\alpha 6/\beta 3$  combination plays such a large role in TETS action on the GABA<sub>A</sub> receptor. Of these two  $\beta$  subunits,  $\beta 2$  and  $\beta 3$ , that are able to participate in TETS binding, the more sensitive  $\beta 3$  subunit is probably more important for the proconvulsant activity of TETS. Mice lacking the  $\beta 3$  subunit display features reminiscent of Angelman syndrome in humans including abnormal EEG with interictal spikes and slowing, seizures, hyperactivity, impaired learning and memory and repetitive behavior (DeLorey et al. 1998; Handforth et al. 2005). In contrast, mice lacking  $\beta 2$  do not exhibit spontaneous seizures but are

less susceptible to the hypnotic actions of ethanol and the effects of benzodiazepines (Blednov et al. 2003; Sur et al. 2001).

While TETS and PTX thus differ in their GABA<sub>A</sub> receptor subtype selectivity, with TETS most likely exerting its major action on  $\alpha 2\beta 3\gamma 2$  receptors while PTX does not demonstrate any significant receptor selectivity, we hesitate to use this selectivity as an explanation for the greater *in vivo* toxicity of TETS since the differences in potency could possibly also be pharmacokinetic in nature. When administered intraperitoneally in mice, TETS induces clonic seizures with an ED<sub>50</sub> of 0.14 mg/kg and is lethal at a dose of 0.3 mg/kg, which is roughly 40 times more potent than picrotoxin (Zolkowska et al. 2012). However, when TETS and picrotoxin (which in this case contained only 50% of the active picrotoxinin) were administered intraventricularly, they were found to be equipotent in their ability to induce convulsions (Zolkowska et al. 2012) suggesting that the higher potency of TETS when administered systemically could be due to brain penetration or brain uptake than PTX. Another possibility is of course that TETS has additional actions on other receptors than GABA<sub>A</sub> receptors that promote seizures and convulsions.

Both TETS and PTX failed to fully block GABA-induced chloride currents through all tested GABA<sub>A</sub> receptor subunit combinations under our experimental conditions in which we elicited near maximal currents by using GABA EC<sub>90</sub> concentrations for each receptor, and could typically only achieve 70-85% of block. It is of course possible that we could have achieved some additional block by applying millimolar TETS or PTX concentrations, but we chose to not test concentrations higher than 100  $\mu$ M of TETS on most receptors since we regard these high concentrations as physiologically unrealistic. However, it should be mentioned here that the remaining current could be blocked by bicuculline (100  $\mu$ M) or fipronil (10  $\mu$ M). Out of these two compounds, bicuculline is known to competitively inhibit GABA binding at the orthosteric site, although it also seems to have additional allosteric actions (Johnston 2013), while the phenylpyrazole fipronil has been shown to bind to the same site as picrotoxinin and several polychlorocycloalkane insecticides in radioligand binding assays on the

homopentameric  $\beta 3$  receptor (Chen et al. 2006). This of course raises the question of the TETS binding site, which based on the non-competitive nature of its inhibition, and the observations that TETS displaces pore blockers like [ $^{35}\text{S}$ ]TBPS (Esser et al. 1991; Squires et al. 1983) and the cage convulsant EBOB (Ratra et al. 2001; Zhao et al. 2014), is most likely located in the pore region of the channel, where TETS has been predicted by molecular dynamics simulations to hydrogen bond with two  $\alpha 1$ -M2 segments and one  $\gamma 2$ -M2 segment in a homology model of the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor. This model (Zhao et al. 2014), which was based on the crystal structure of the homopentameric *Caenorhabditis elegans* glutamate-gated chloride channel (Hibbs and Gouaux 2011), and which has never been probed by mutagenesis, showed TETS making no contacts with the  $\beta$  subunit, thus, apparently providing an explanation for why the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptor is sensitive to TETS, while the  $\beta 3$  homopentamer is not (Ratra et al. 2001). This molecular model should be re-examined in light of our findings that TETS shows the highest potency for blocking chloride current through GABA<sub>A</sub> receptors containing  $\alpha 2$  or  $\alpha 6$  as well as  $\beta 3$  subunits. Ideally, the TETS binding site should be mapped through a combination of site-directed mutagenesis and molecular modeling using new homology models of the  $\alpha 2\beta 3\gamma 2$ ,  $\alpha 2\beta 2\gamma 2$  and the  $\alpha 1\beta 2\gamma 2$  GABA<sub>A</sub> receptors based on the more recently crystallized human  $\beta 3$  homopentamer (Miller and Aricescu 2014).

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doi:10.1124/jpet.111.190579

**Table 1****Pharmacological signature of expressed GABA<sub>A</sub> receptor isoforms**

GABA <sub>A</sub> Isoform	Sensitivity to Positive allosteric modulators	Sensitivity to Inhibitors
α1β1γ2L	Diazepam <sup>+</sup> , Propofol <sup>+</sup> , Allopregnanolone <sup>+</sup>	SAS <sup>+</sup> , Bicuculline <sup>+</sup> , Fipronil <sup>+</sup>
α1β2γ2L	Diazepam <sup>+</sup> , Propofol <sup>+</sup> , Allopregnanolone <sup>+</sup>	Fipronil <sup>+</sup>
α1β3γ2L	Diazepam <sup>+</sup> , Propofol <sup>+</sup> , Allopregnanolone <sup>+</sup>	Fipronil <sup>+</sup>
α2β2γ2L	Diazepam <sup>+</sup> , Propofol <sup>+</sup>	Fipronil <sup>+</sup>
α2β3γ2L	Diazepam <sup>+</sup> , Propofol <sup>+</sup> , Allopregnanolone <sup>+</sup>	SAS <sup>neg</sup> , Bicuculline <sup>+</sup> , Fipronil <sup>+</sup> , Zn <sup>2+neg</sup>
α2β3	Diazepam <sup>neg</sup> , Propofol <sup>+</sup> ,	Fipronil <sup>+</sup>
α4β3γ2L	Diazepam <sup>neg</sup> , Propofol <sup>+</sup> ,	Fipronil <sup>+</sup>
α4β3δ	Diazepam <sup>neg</sup> , Allopregnanolone <sup>+</sup> , DS2 <sup>+</sup>	Fipronil <sup>+</sup>
α6β1γ2L	Diazepam <sup>neg</sup>	SAS <sup>+</sup> , Bicuculline <sup>+</sup> , Fipronil <sup>+</sup>
α6β2γ2L	Diazepam <sup>neg</sup> , Propofol <sup>+</sup>	Fipronil <sup>+</sup>
α6β3γ2L	Diazepam <sup>neg</sup> , Propofol <sup>+</sup> , Allopregnanolone <sup>+</sup>	SAS <sup>neg</sup> , Bicuculline <sup>+</sup> , Fipronil <sup>+</sup> , Zn <sup>2+neg</sup>
α6β3	ND	Zn <sup>2+</sup> positive
α6β3γ1	Diazepam <sup>neg</sup> , Propofol <sup>+</sup>	Fipronil <sup>+</sup>
α6β3δ	Diazepam <sup>neg</sup> , DS2 <sup>+</sup>	Fipronil <sup>+</sup>

Sensitivity to 10  $\mu$ M diazepam, 250 nM Allopregnanolone, 100  $\mu$ M Propofol, 50  $\mu$ M DS2 (4-chloro-*N*-[2-(2-thienyl)imidazo[1,2-*a*]pyridin-3-yl]benzamide), 10  $\mu$ M Fipronil, 100  $\mu$ M Bicuculline, 10  $\mu$ M SAS (salicylidene salicylhydrazide) and 10  $\mu$ M ZnCl<sub>2</sub> was tested on 3-10 cells per subunit combination. ND = Not determined.

**Table 2****IC<sub>50</sub> values for TETS and PTX**

GABA <sub>A</sub> Isoform	TETS ( $\mu$ M)	95% CI	PTX ( $\mu$ M)	95% CI
$\alpha$ 1 $\beta$ 1 $\gamma$ 2L	> 20	-	> 30	-
$\alpha$ 1 $\beta$ 2 $\gamma$ 2L	3.60	2.1-4.1	7.98	6.58-9.38
$\alpha$ 1 $\beta$ 3 $\gamma$ 2L	3.41	1.4-5.4	3.7	2.1-5.3
$\alpha$ 2 $\beta$ 2 $\gamma$ 2L	12.2	10.5-15.8	11	8.6-13.5
$\alpha$ 2 $\beta$ 3 $\gamma$ 2L	0.48	0.32-0.64	7.5	5.2-9.9
$\alpha$ 2 $\beta$ 3	3.37	2.77-3.97	1.98	0.67-3.29
$\alpha$ 4 $\beta$ 3 $\gamma$ 2L	1.82	1.02-2.62	3.0	1.7-4.3
$\alpha$ 4 $\beta$ 3 $\delta$	2.66	1.36-3.96	ND	ND
$\alpha$ 6 $\beta$ 1 $\gamma$ 2L	> 20	-	ND	ND
$\alpha$ 6 $\beta$ 2 $\gamma$ 2L	20	18.3-21.7	26.9	20.3-33.2
$\alpha$ 6 $\beta$ 3 $\gamma$ 2L	0.40	0.29-0.51	5.8	4.2-7.4
$\alpha$ 6 $\beta$ 3 $\gamma$ 1	6.81	3.9-9.7	ND	ND
$\alpha$ 6 $\beta$ 3 $\delta$	3.69	3.18-4.2	ND	ND

ND = Not determined

## Figure Legends

**Fig. 1** Chemical structures of TETS and picrotoxinin.

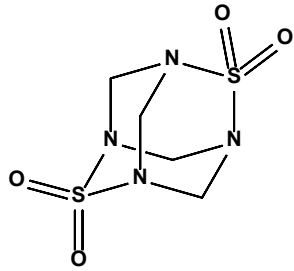
**Fig. 2** GABA concentration-response curves for  $\alpha 1$  (A),  $\alpha 2$  (B),  $\alpha 4$  (C) and  $\alpha 6$  (D) containing GABA<sub>A</sub> receptor combinations. Individual data points are presented as mean  $\pm$  SD from 7-20 independent recordings. EC<sub>50</sub> values are presented with 95% confidence intervals.

**Fig. 3** (A) Concentration-response curves comparing TETS inhibition of different  $\beta$  subunits in the  $\alpha 1\beta X\gamma 2L$  combination. (B) Comparison of TETS inhibition of  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 4$  and  $\alpha 6$  subunits in  $\alpha X\beta 3\gamma 2L$  containing GABA<sub>A</sub> receptor combinations. Individual data points are presented as mean  $\pm$  SD from 3-8 independent recordings. EC<sub>50</sub> values are presented with 95% confidence intervals. Example traces showing TETS inhibition of  $\alpha 2\beta 3\gamma 2L$  (C) and  $\alpha 6\beta 3\gamma 2L$  (D) receptors. EC<sub>90</sub> GABA was applied first alone as control and then in the presence of 1  $\mu M$  or 100  $\mu M$  of TETS.

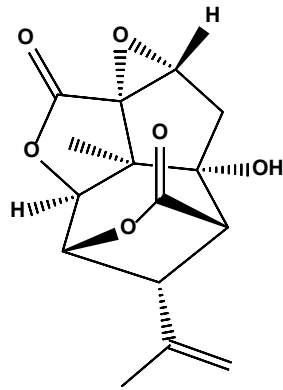
**Fig. 4** (A) Concentration-response curves for TETS inhibition of currents evoked by EC<sub>90</sub> GABA for  $\alpha 2\beta X\gamma 2L$  receptors to determine the changes induced by exchanging  $\beta 2$  for  $\beta 3$ . The concentration response curve of TETS for  $\alpha 2\beta 2\gamma 2L$  (IC<sub>50</sub> 12.2  $\mu M$ , 95% CI 10.5-15.8  $\mu M$ , Emax ~65%) was right shifted in comparison to  $\alpha 2\beta 3\gamma 2L$  (IC<sub>50</sub> 480 nM, 95% CI 10.5-15.8 nM, Emax ~80%). (B) Both the  $\alpha 6\beta 1\gamma 2L$  receptor (IC<sub>50</sub> >20  $\mu M$ , Emax ~25%) and the  $\alpha 6\beta 2\gamma 2L$  receptor (IC<sub>50</sub> 20  $\mu M$ , 95% CI 18.3-21.7  $\mu M$ , Emax ~35%) showed a significantly reduced response to TETS. The  $\alpha 6\beta 3\gamma 2L$  receptor (IC<sub>50</sub> 400 nM, 95% CI 290-510 nM, Emax ~70%) is highly sensitive to TETS as shown previously in Fig. 3. (C) The  $\alpha 4\beta 3\gamma 2L$  receptor (IC<sub>50</sub> 1.82  $\mu M$ , 95% CI 1.02-2.62  $\mu M$ , Emax ~46%,  $n_H = 0.7$ ) showed a reduced Emax for TETS inhibition, while the  $\alpha 4\beta 3\delta$  receptor (IC<sub>50</sub> 3  $\mu M$ , 95% CI 1.36-3.96  $\mu M$ , Emax ~85%,  $n_H = 1.2$ ) showed an increased Hill coefficient. (D) In  $\alpha 6$  containing receptors ( $\alpha 6\beta 3\gamma 2L$ : IC<sub>50</sub> 400 nM, 95%

CI 0.29-0.51 nM Emax ~70% as previously shown) introduction of a  $\delta$  subunit increased Emax and reduced potency ( $\alpha 6\beta 3\delta$ : IC<sub>50</sub> 3.69  $\mu$ M, 95% CI 3.18-4.2  $\mu$ M, Emax ~85%), while replacement of  $\gamma 2L$  with  $\gamma 1$  right-shifted the concentration response curve ( $\alpha 6\beta 3\gamma 1$ : IC<sub>50</sub> 6.81  $\mu$ M, 95% CI 3.9-9.7  $\mu$ M, Emax ~65%). Individual data points are presented as mean  $\pm$  SD from 3-9 independent recordings. EC<sub>50</sub> values are presented with 95% confidence intervals. Please note that the  $\alpha 4\beta 3\delta$  receptor combination was stably expressed in Lt-K cells while the  $\alpha 6\beta 3\delta$  combination was transiently expressed in COS-7 cells.

**Fig. 5** Concentration-response curves for PTX inhibition of  $\beta 1/\beta 2$  (A) and  $\beta 3$  (B) containing GABA<sub>A</sub> receptor combinations. (C) Effect of TETS (2.5  $\mu$ M) and PTX (10  $\mu$ M) at roughly IC80 concentration on the GABA concentration response curve of the  $\alpha 2\beta 3\gamma 2L$  receptor. Individual data points are presented as mean  $\pm$  SD 3-8 independent recordings. EC<sub>50</sub> values are presented with 95% confidence intervals.



**TETS**



**Picrotoxinin (PTX)**

**Figure 1**

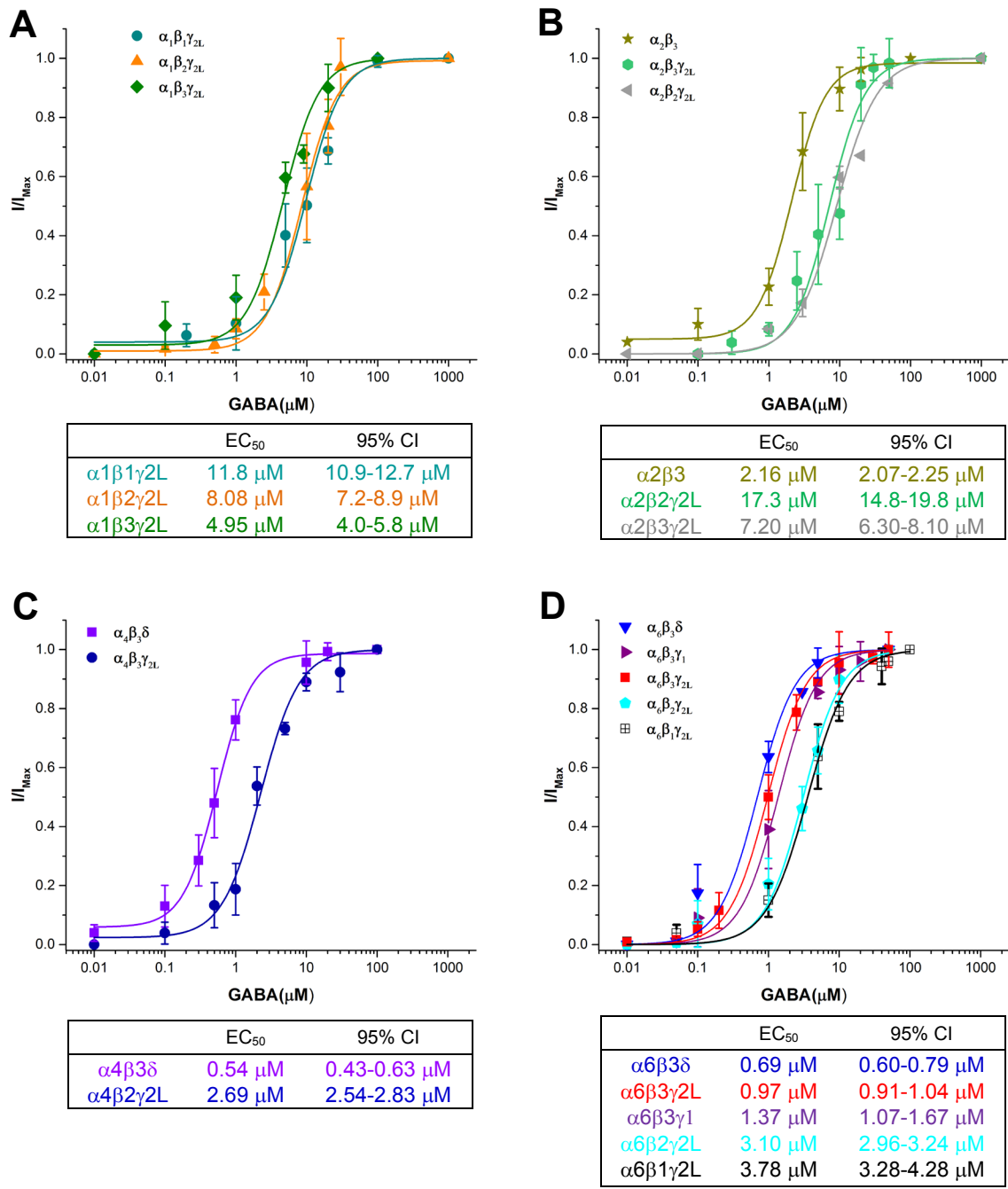
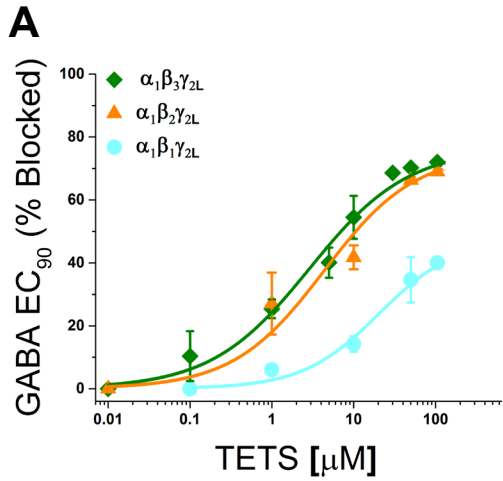
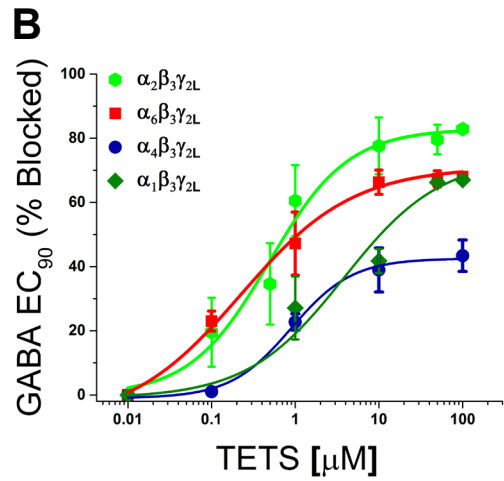


Figure 2





	IC <sub>50</sub>	95% CI
$\alpha_1\beta_3\gamma_{2L}$	3.41 $\mu\text{M}$	1.40-5.40 $\mu\text{M}$
$\alpha_1\beta_2\gamma_{2L}$	3.60 $\mu\text{M}$	2.10-4.10 $\mu\text{M}$
$\alpha_1\beta_1\gamma_{2L}$	Emax < 50% at 100 $\mu\text{M}$	



	IC <sub>50</sub>	95% CI
$\alpha_6\beta_3\gamma_{2L}$	0.40 $\mu\text{M}$	0.29-0.51 $\mu\text{M}$
$\alpha_2\beta_3\gamma_{2L}$	0.48 $\mu\text{M}$	0.32-0.64 $\mu\text{M}$
$\alpha_1\beta_3\gamma_{2L}$	3.41 $\mu\text{M}$	1.40-5.40 $\mu\text{M}$
$\alpha_4\beta_3\gamma_{2L}$	Emax < 50% at 100 $\mu\text{M}$	

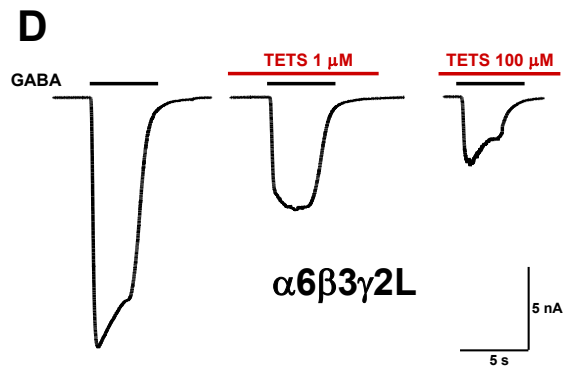
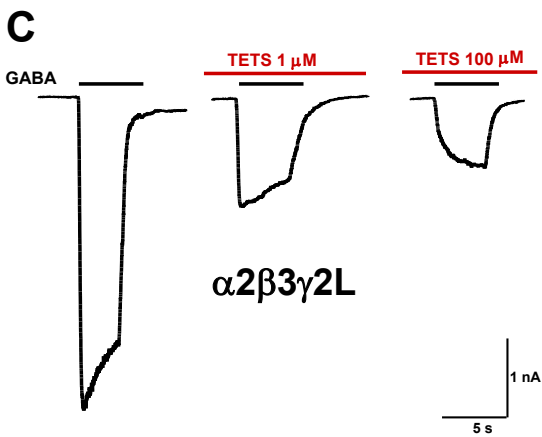


Figure 3

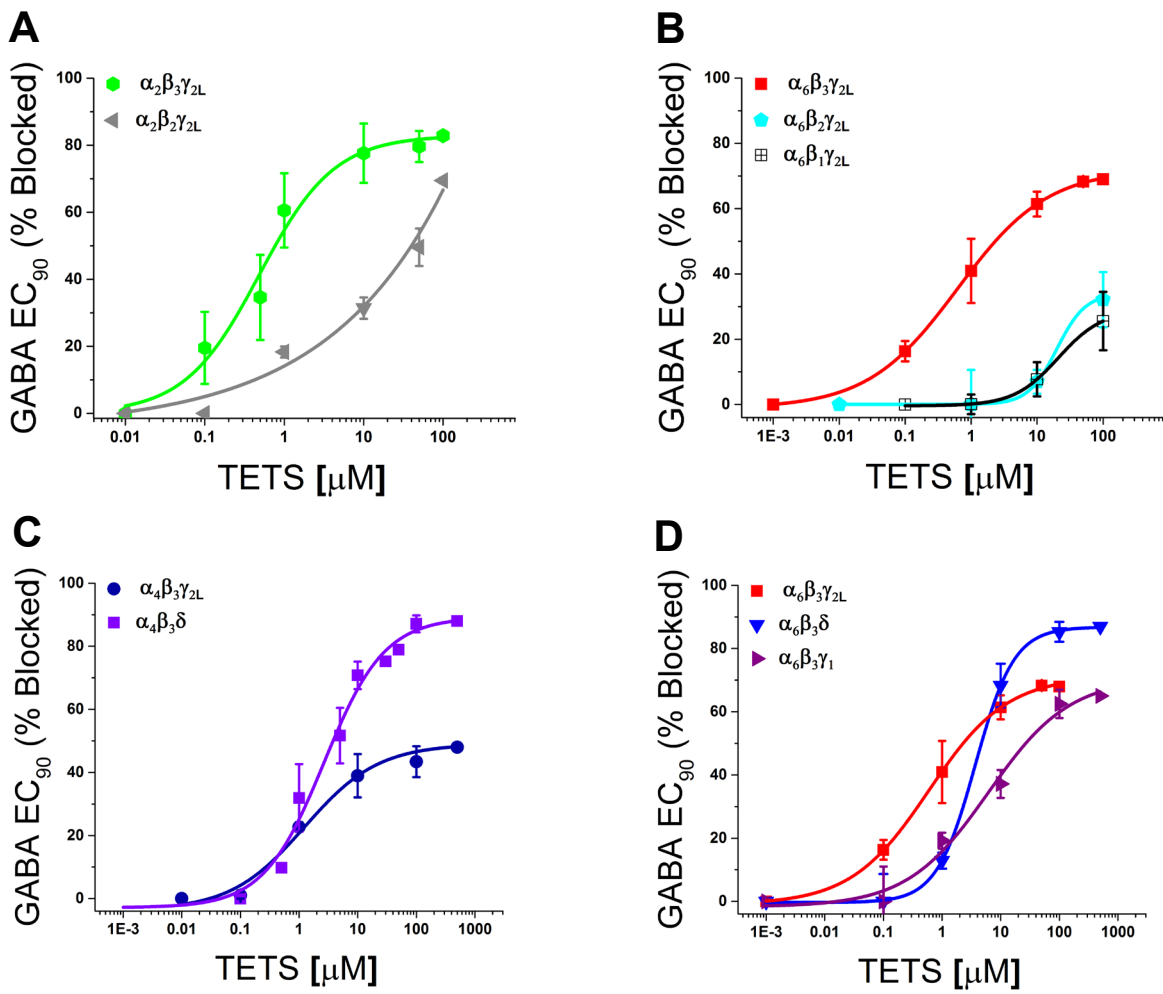
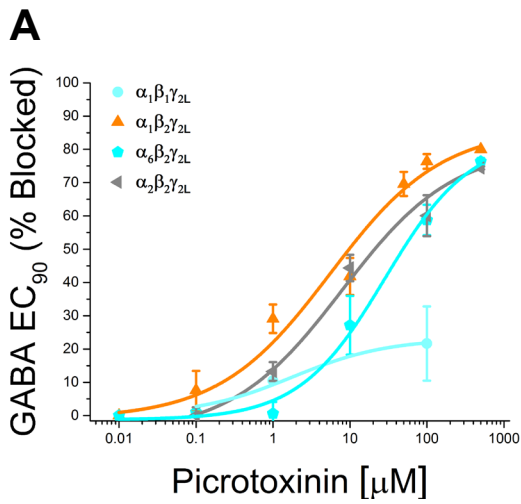
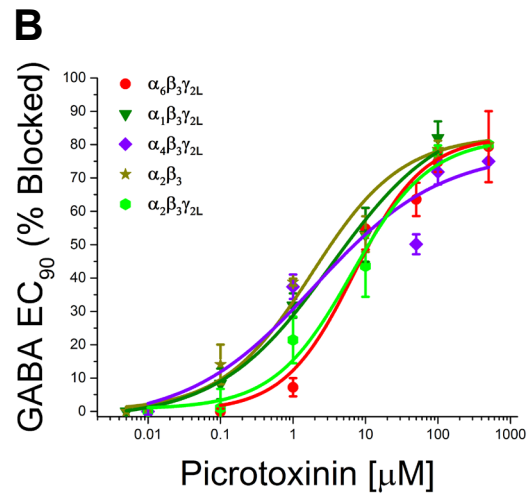


Figure 4



	EC <sub>50</sub>	95% CI
$\alpha_1\beta_1\gamma_2\text{L}$	E <sub>max</sub> <50% at 100 $\mu\text{M}$	
$\alpha_1\beta_2\gamma_2\text{L}$	7.98 $\mu\text{M}$	6.58-9.38 $\mu\text{M}$
$\alpha_6\beta_2\gamma_2\text{L}$	26.9 $\mu\text{M}$	20.3-33.2 $\mu\text{M}$
$\alpha_2\beta_2\gamma_2\text{L}$	11 $\mu\text{M}$	8.6-13.5 $\mu\text{M}$



	EC <sub>50</sub>	95% CI
$\alpha_6\beta_3\gamma_2\text{L}$	5.8 $\mu\text{M}$	4.2-7.4 $\mu\text{M}$
$\alpha_1\beta_3\gamma_2\text{L}$	3.7 $\mu\text{M}$	2.1-5.3 $\mu\text{M}$
$\alpha_4\beta_3\gamma_2\text{L}$	3.0 $\mu\text{M}$	1.7-4.3 $\mu\text{M}$
$\alpha_2\beta_3$	1.98 $\mu\text{M}$	0.67-3.29 $\mu\text{M}$
$\alpha_2\beta_3\gamma_2\text{L}$	7.5 $\mu\text{M}$	5.2-9.9 $\mu\text{M}$

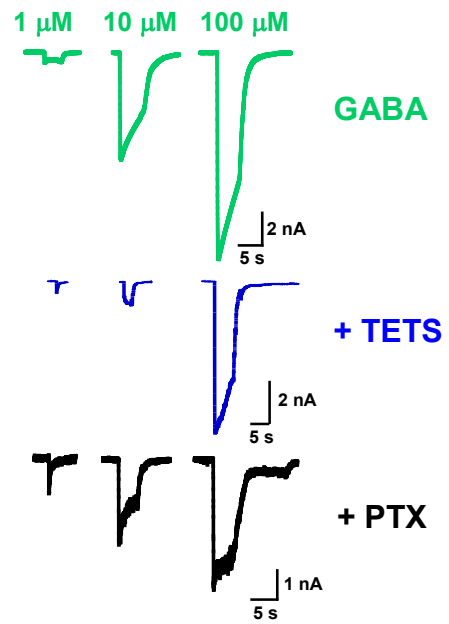
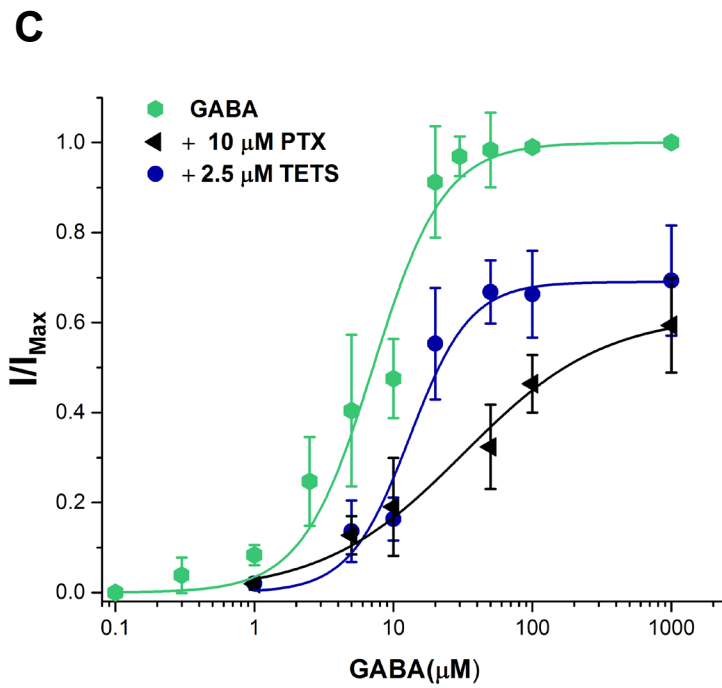


Figure 5