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GABA_A receptor target of tetramethylenedisulfotetramine

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Contributed by John E. Casida, April 24, 2014 (sent for review March 22, 2014)

Use of the highly toxic and easily prepared rodenticide tetramethylenedisulfotetramine (TETS) was banned after thousands of accidental or intentional human poisonings, but it is of continued concern as a chemical threat agent. TETS is a noncompetitive blocker of the GABA type A receptor (GABA_AR), but its molecular interaction has not been directly established for lack of a suitable radioligand to localize the binding site. We synthesized [¹⁴C]TETS (14 mCi/mmol, radiochemical purity >99%) by reacting sulfamide with H¹⁴CHO and *s*-trioxane then completion of the sequential cyclization with excess HCHO. The outstanding radiocarbon sensitivity of accelerator mass spectrometry (AMS) allowed the use of [¹⁴C]TETS in neuroreceptor binding studies with rat brain membranes in comparison with the standard GABA_AR radioligand 4'-ethynyl-4-*n*-[³H]propylbicycloorthobenzoate ([³H]EBOB) (46 Ci/mmol), illustrating the use of AMS for characterizing the binding sites of high-affinity ¹⁴C radioligands. Fourteen noncompetitive antagonists of widely diverse chemotypes assayed at 1 or 10 μM inhibited [¹⁴C]TETS and [³H]EBOB binding to a similar extent (*r*² = 0.71). Molecular dynamics simulations of these 14 toxicants in the pore region of the α1β2γ2 GABA_AR predict unique and significant polar interactions for TETS with α1T1' and γ2S2', which are not observed for EBOB or the GABAergic insecticides. Several GABA_AR modulators similarly inhibited [¹⁴C]TETS and [³H]EBOB binding, including midazolam, flurazepam, avermectin Ba1, baclofen, isoguvacine, and propofol, at 1 or 10 μM, providing an *in vitro* system for recognizing candidate antidotes.

neurotoxicity | convulsant | molecular modeling

Severe poisonings in a German furniture factory in the 1940s were traced to wool impregnated with the resinous reaction product of sulfamide (NH₂SO₂NH₂) and formaldehyde (HCHO). The causative agent was identified as tetramethylenedisulfotetramine (TETS, also known as tetramine) which was then developed as a rodenticide (now illegal) and continues to be of concern as a chemical threat agent. The chronology of TETS chemistry and toxicology is given briefly here and more extensively in *SI Appendix, section S1*.

TETS was first synthesized more than 80 y ago (1–3). Structure–activity studies showed that any structural modification greatly reduces the toxicity (4). The need to understand the distribution and fate of TETS led to ¹⁴C radiosynthesis in 1967 (5) by an undisclosed method, but the product was only 80% pure, limiting the interpretation of biological experiments. Analysis is achieved by liquid chromatography/MS (6) or when ultrahigh sensitivity is required and [¹⁴C]TETS is available by accelerator mass spectrometry (AMS) (7), as reported here.

TETS is highly toxic to mammals with an *i.p.* LD₅₀ of 0.11–0.22 mg/kg in mice and rats, leading to its use as a rodenticide until it was banned worldwide in the early 1990s (2, 8, 9). However, it is still available illegally and responsible for accidental or intentional poisonings in China and other countries. The estimated lethal dose of 7–10 mg in adult humans coupled with its ease of synthesis and stability serve as the basis for the chemical threat concern (10–15). Neurotoxicity is sometimes alleviated or antidoted by compounds

modulating the target site to reduce disruption by the toxicant. TETS toxicity is reported to be alleviated in rodents or humans by diazepam, barbiturates, allopregnanolone, and sodium 2,3-dimercapto-1-propanesulfonate (NaDMPS), some of which are GABA_AR modulators (16–24) (*SI Appendix, section S1*). TETS is one of several small-cage convulsants, a group that also includes bicyclopophosphorus compounds, such as the even more toxic *t*-butylbicyclopophosphate (TBPO) and *t*-butylbicyclopophosphorothionate (TBPS) (25) (Fig. 1).

TETS is a noncompetitive antagonist of the GABA type A receptor (GABA_AR) based on multiple physiological and toxicological criteria* (20, 26–29) (*SI Appendix, section S1*) and GABA_AR assays with two trioxabicyclooctane radioligands, [³⁵S]TBPS (30), and 4-*n*-[³H]propyl-4'-ethynylbicycloorthobenzoate ([³H]EBOB) (31) (Fig. 1). TETS is a competitive inhibitor of [³H]EBOB binding with a potency in rat brain GABA_AR consistent with its toxicity in mice (4, 32). However, it does not inhibit human GABA_AR recombinant β₃ homopentamer assayed with [³H]EBOB (33), which has a structure–activity relationship for inhibitors similar to that for the housefly GABAR (34). These deductions are based on the use of [³⁵S]TBPS and [³H]EBOB to assay the action of TETS.

Direct observation of the TETS binding site requires the use of TETS as the radioligand. Radioligand binding studies for neuroreceptors as toxicant targets normally require high specific activities (>10 Ci/mmol) such as ³H labeling, which is not available to date for TETS. [¹⁴C]TETS reported here has a specific activity

Significance

Tetramethylenedisulfotetramine (TETS) is a feared chemical threat agent because of its high convulsant toxicity, ease of synthesis, and availability even though it is banned as a rodenticide. Earlier physiological evidence indicating action as a GABA receptor antagonist and inhibitor of [³⁵S]TBPS and [³H]EBOB binding is confirmed here by radiosynthesis of [¹⁴C]TETS and defining its binding site in rat brain membranes by accelerator mass spectrometry and toxicant specificity studies on inhibition of [¹⁴C]TETS and [³H]EBOB binding. TETS undergoes specific and unique polar interactions inside the 1'2' ring pore region instead of the 2',6', and 9' site for insecticides. This study helps define GABA_AR sites for potential antidotes acting to prevent TETS binding or displace it from its binding site.

Author contributions: B.D.H. and J.E.C. designed research; C.Z., S.H.H., B.A.B., and J.Y. performed research; T.S.C. and F.C.L. analyzed data; and B.D.H. and J.E.C. wrote the paper. The authors declare no conflict of interest.

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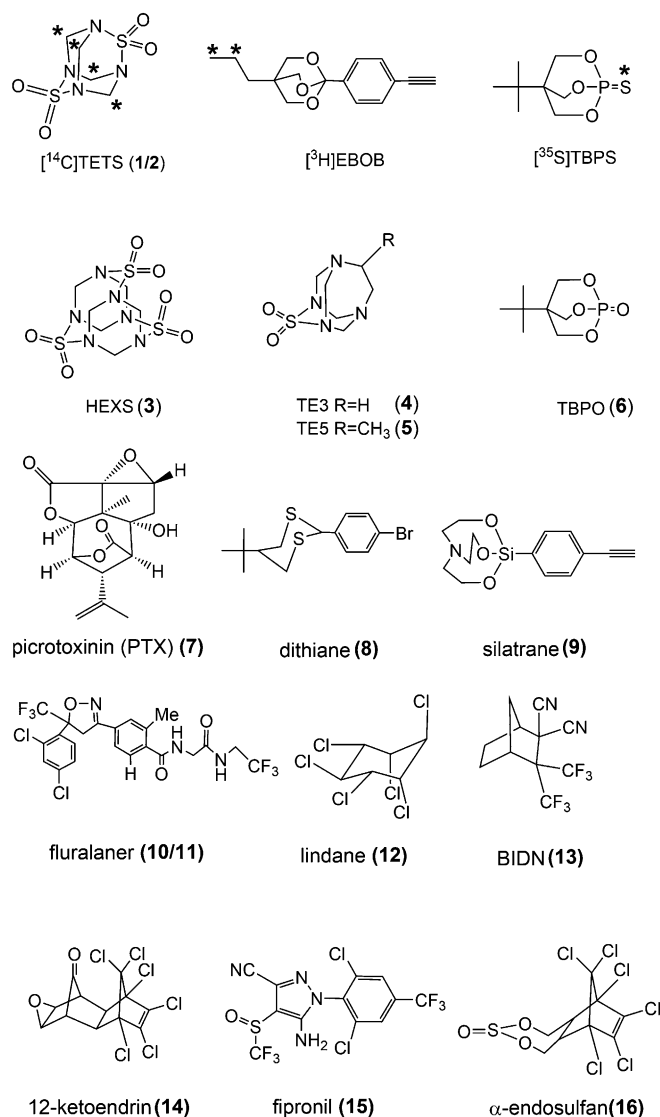


Fig. 1. Structures of three radioligands and unlabeled insecticides or convulsants with number designations. Two numbers for TETS (1/2) and fluralaner (10/11) refer to different concentrations considered later.

of 14 mCi/mmol, which is only about 0.02–0.1% the level normally used for radioligand binding assays. The required detection could only be achieved with [^{14}C]TETS by greatly enhanced sensitivity resulting from AMS, which to our knowledge has never been used before in neuroreceptor radioligand binding assays.

This study characterizes the [^{14}C]TETS binding site in rat brain GABA_AR . TETS and EBOB are compared as an unknown versus a standard cage convulsant radioligand that may have some common features in their binding sites. Rat brain is used because TETS is primarily a rodenticide. The same experiments are run with [^{14}C]TETS and [^3H]EBOB so that only the radioligand and analytical method are varied to best evaluate the utility of the new radioligand and the AMS analysis technique in defining the mechanism of TETS toxicity. The [^{14}C]TETS binding assay allows verification of the mode of action, definition of the pharmacological profile, localization of the binding site, and characterization of potential antidotes or alleviating agents.

Results and Discussion

Synthesis of [^{13}C]TETS and [^{14}C]TETS. Unlabeled TETS is readily synthesized from sulfamide by reacting with HCHO (37% wt/vol

in water) or its equivalent such as *s*-trioxane or paraformaldehyde in acidic condition (2, 4). However, the low concentration of commercially available H^{14}CHO (1–3% in water) seriously delayed the final ring cyclization step to form [^{14}C]TETS. To overcome this, the procedure was modified by stepwise cyclization (Fig. 2). Reaction conditions were optimized through tests with H^{13}CHO as the H^{14}CHO mimic. H^{13}CHO (20% aqueous solution, 0.25 equivalents relative to sulfamide) and *s*-trioxane (source of 1.75 equivalents of HCHO as a solid form) ensured that all H^{13}CHO was incorporated into the product owing to the slower release of unlabeled HCHO from *s*-trioxane. An additional treatment with unlabeled HCHO completed the final cyclization reaction. These conditions were then used to prepare the [^{14}C]TETS as follows. To a chilled solution of sulfamide (1.9 mg, 20 μmol) and *s*-trioxane (1.1 mg, 12 μmol) in 21 μL of concentrated hydrochloric acid (conc. HCl, 250 μmol) was added 250 μL of H^{14}CHO (5 μmol , 3% in water, 250 μCi , specific activity 50 mCi/mmol, 99% pure by HPLC) at 0 $^\circ\text{C}$. The reaction mixture was slowly warmed to room temperature and stirred for 1 d. After adding acetonitrile (250 μL), the azeotrope was evaporated under a stream of dry air at room temperature. To the remaining reaction mixture were added 21 μL of conc. HCl and 3.8 μL of unlabeled HCHO (37% in water). After 1 h, acetonitrile (100 μL) was added and the azeotrope was again removed under a stream of dry air at room temperature. The remaining solid was dissolved by adding 100 μL of acetone then 500 μL of dichloromethane, yielding a white precipitate. After filtration, the filtrate was evaporated under a stream of dry air and the residual crude TETS was purified by column chromatography on silica gel with an eluent (dichloromethane: *n*-hexane 3:1, $R_f = 0.54$). The [^{14}C]TETS was obtained on evaporation as a white solid: 310 μg , 12.9% chemical yield, 7.2% radiochemical yield, specific activity 14 mCi/mmol, and >99% radiochemical purity (*SI Appendix, section S2*). The product was dissolved in 1 mL acetone and stored in a sealed amber glass ampoule at $-20\text{ }^\circ\text{C}$. GC-MS analysis data for the final [^{13}C]TETS and [^{14}C]TETS revealed slightly less label incorporation with [^{14}C]TETS (Table 1).

[^{14}C]TETS Binding Parameters. Neuroreceptor binding assays with a ^{14}C -labeled compound require an ultrasensitive analytical method provided by the use of tandem HPLC and AMS with a typical limit of quantification of 2–20 amol, which proved to be adequate in the present studies.

[^{14}C]TETS undergoes specific binding to rat brain membranes at 37 $^\circ\text{C}$ with half saturation at 0.08 μM (Fig. 3A). TETS is also a potent inhibitor of [^3H]EBOB binding under the same conditions with an IC_{50} of 0.79 μM (Fig. 3B). Nonspecific binding was determined with unlabeled TETS at 10 μM . Total, nonspecific, and specific binding with [^{14}C]TETS were 1,390, 454, and 1,019 fg TETS/ μg protein, respectively (i.e., $67 \pm 1\%$ specific binding). The corresponding values with [^3H]EBOB were 2,013, 994, and 1,019 dpm/125 μg protein, respectively, corresponding to $50 \pm 5\%$ specific binding. GABA at 0.3, 1, and 10 μM inhibited [^{14}C]TETS binding by 15 ± 3 , 55 ± 1 , and $79 \pm 3\%$, respectively, and the corresponding values for [^3H]EBOB were 42 ± 8 , 61 ± 4 , and

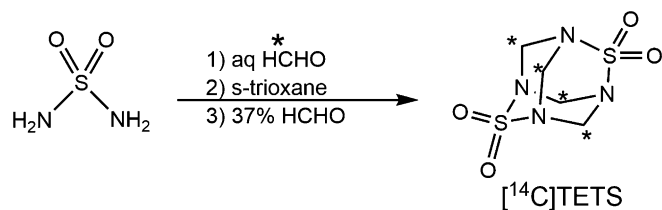


Fig. 2. Synthesis of [^{13}C]TETS and [^{14}C]TETS by serial cyclization steps in concentrated hydrochloric acid. The percentages of mono-, di-, tri-, and tetra-labeling are given in Table 1.

Table 1. Labeling of [¹³C]TETS and [¹⁴C]TETS from 20% aqueous H¹³CHO and 3% aqueous H¹⁴CHO (50 mCi/mmol) and specific activity of [¹⁴C]TETS

No. of labeled carbons	[¹³ C]TETS, %*	[¹⁴ C]TETS		
		%*	mCi/mmol [†]	Contribution [‡]
0	67.3	76.2	0	0
1	25.4	19.8	50	9.9
2	5.7	3.4	100	3.4
3	1.2	0.6	150	0.9
4	0.4	0	200	0
Total	100	100		14.2 [§]

*Percentage isotopic distribution of TETS determined by GC-MS in full-scan mode for [¹³C]TETS and selected ion monitoring mode for [¹⁴C]TETS (*SI Appendix, section S2*).

[†]Theoretical specific activity of TETS with the indicated number of ¹⁴C-labeled carbon(s).

[‡]Contribution of components with one, two, and three ¹⁴C-labeled carbon(s) summed to give the total specific activity (mCi/mmol) of the final [¹⁴C]TETS.

[§]Experimental specific activity (14.0 mCi/mmol) of the final [¹⁴C]TETS was determined by liquid scintillation counting of 1 μL aliquot from 1 mL acetone solution of 310 μg [¹⁴C]TETS.

104 ± 2%, respectively. L-glutamic acid did not inhibit binding of either radioligand at 1 μM.

Convulsants and Insecticides Compete Similarly for [¹⁴C]TETS and [³H]EBOB Binding Sites. If [¹⁴C]TETS and [³H]EBOB bind at the same site in the same way (i.e., are superimposable), they should be similarly inhibited by a series of convulsants and insecticides selected for their widely varied chemotypes and assayed at 1 or 10 μM. The results for 16 compounds or concentrations (Fig. 1) are presented in Fig. 4 and *SI Appendix, section S3*. Earlier published findings on the [³H]EBOB and [³⁵S]TBPS sites are given in *SI Appendix, section S4*. TETS with an IC₅₀ of 0.08 μM (Fig. 3A) was considerably more potent than three of its analogs assayed with either radioligand. The TETS-type compounds (1–5) and several insecticides or cage convulsants (6–16) inhibit [¹⁴C]TETS and [³H]EBOB binding to a similar extent ($r^2 = 0.71$). TETS, EBOB, and the other convulsants and insecticides therefore compete with each other at comparable binding sites, prompting atomistic, structural examination in the GABA_AR pore.

Different Binding Positions for TETS and EBOB. Much is known about the binding sites for EBOB, picrotoxinin (PTX), lindane, 3,3-bis-trifluoromethyl-bicyclo[2,2,1]heptane-2,2-dicarbonitrile, fipronil, and α-endosulfan in the α₁β₂γ₂ GABA_AR (33–40). TETS, TBPO, and EBOB were initially molecularly docked in the pore region of

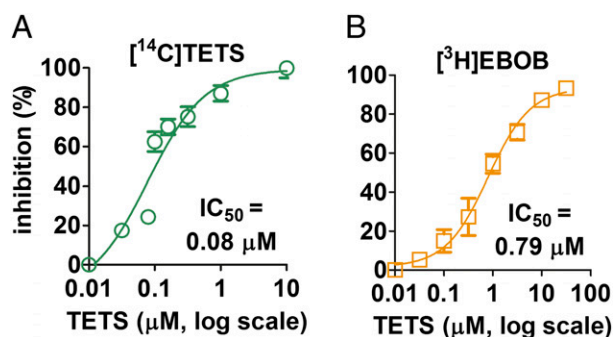


Fig. 3. TETS target assayed as (A) displacement of [¹⁴C]TETS and (B) inhibition of [³H]EBOB binding in rat brain membranes.

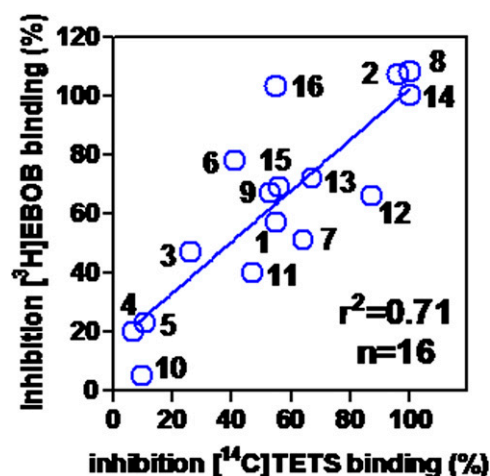


Fig. 4. Inhibition of [¹⁴C]TETS and [³H]EBOB binding in rat brain membranes by convulsants and insecticides at 10 μM (2 and 11) or 1 μM (all other data). Plotted from data in *SI Appendix, section S3*.

the α₁β₂γ₂ GABA_AR model. After 40 ns of molecular dynamics (MD) simulations (described in *SI Appendix, section S5*), their optimized and equilibrated positions (Fig. 5) illustrate that EBOB and TBPO overlap the proposed TETS binding site (located around the 1'2' region of the pore). MD simulations of all of the insecticides and convulsants in this study predict a partially overlapping binding site with a common region at the 1'2' position (the 2' "contact zone") (*SI Appendix, section S6*). However, the interactions with specific residues in the 1'2' position can differ. TETS and EBOB, for example, both make a substantial proportion of all their calculated contacts to the 1'2' residues (61% and 45%, respectively). However, whereas EBOB contacts the αγ subunits at the 1'2' site 68% of the time (only slightly more than the 60% expected for nonspecific subunit binding), TETS contacts the αγ subunits at the 1'2' site 98% of the time with an α₁:β₂ ratio of 31:1, suggesting a specific α₁ interaction. Moreover, 69% of all of the simulated TETS contacts with GABA_AR are made to just the α₁T1' and γ₂S2' residues. In contrast, EBOB makes only 6% of its contacts to these residues. Extracting detailed interactions from our simulations posits four ways to interact with the 1'2' residues (*SI Appendix, section S7*): specific polar interactions (TETS), both significant polar and hydrophobic interactions (TBPO and TBPS), general hydrophobic interactions (such as EBOB), and nonspecific or nonsignificant interactions that imply that other residues within the GABA_AR pore are more important for binding (e.g., PTX interacts with 6' as a key residue).

The predicted TETS and EBOB residue contact differences and binding interactions correspond with the sensitivity and specificity observed in expressed human β₃ (hydrophobic at 1'2') homopentameric GABA_ARs (33). In agreement with mutation studies that show that changes to the β₃ homopentamer 2' residue from hydrophobic to polar (A→S) decreases the affinity of EBOB for the receptor (36, 37, 39), our binding pattern shows EBOB makes significant hydrophobic interactions at the 1'2' region during the simulation. Conversely, our simulated TETS makes polar interactions at this 1'2' region, suggesting that polar residues are needed for TETS binding. In the β₃ subunit valine and alanine have replaced the α₁T1' and γ₂S2' residues, abolishing the necessary polar residues that TETS is predicted to bind, and could explain why α₁β₂γ₂ is sensitive to TETS but the homopentamer is not. Thus, a β₃ homopentamer 1'V→T or 2'A→S mutant (similar to the α₁ or γ₂ residues) may show increased affinity for TETS.

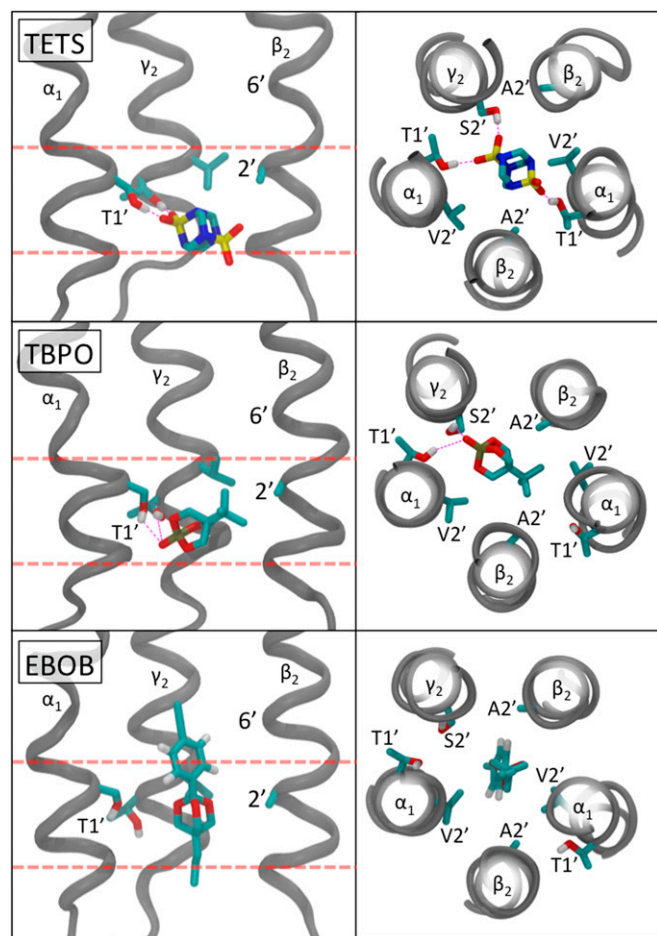


Fig. 5. The equilibrated positions of TETS (**1**), TBPO (**6**), and EBOB following 40 ns of MD simulation in the pore region of the $\alpha_1\beta_2\gamma_2$ GABA_AR model, with views from the side (*Left*, the front two M2 helices have been removed for clarity), and views from the bottom of the pore (*Right*). The red dashed lines signify the common binding region around the 2' "contact zone." The contact zone is the region where a compound can make contacts to 2', either from above or below the residue. The view down the pore shows that at the 1'2' region, TETS makes primarily polar interactions with the α and γ subunits (hydrogen bonds shown as dashed magenta lines), TBPO makes both polar and hydrophobic interactions, and EBOB makes nonspecific hydrophobic interactions with this 1'2' ring of residues.

Types A and B Toxic Action Relative to Binding Positions. TETS and EBOB fall into two different types (A and B) on comparing toxicity with target site potency assayed as inhibition of either [³⁵S]TBPS binding in brain membranes or ³⁶Cl uptake in membrane vesicles of the cerebral cortex (20, 32). Type A compounds include EBOB and many insecticides with large substituents or extended structures, and the type B set includes TETS, TBPS, TBPO, and other small compact molecules, some of very high i.p. toxicity to mice (LD₅₀ 36 μ g/kg for TBPO) (25) (*SI Appendix, section S8*), although much less toxic to injected houseflies (LD₅₀ 90 mg/kg for TETS) (4). The target site mapping studies above suggest a molecular distinction between the binding of type A compounds and Type B cage convulsants (32). Type B antagonists (TETS, TBPO, and TBPS) bind with significant polar interactions, whereas type A antagonists (PTX, lindane, 12-ketoendrin, and EBOB) do not (*SI Appendix, section S7*). In confirmation, distinct differences appear between types A and B compounds on comparing native, $\alpha_1\beta_3\gamma_2$, and $(\beta_3)_5$ GABA_ARs (33). Whereas the type A compounds are exceptionally potent on the β_3 homopentamer, the type B TBPS acts similarly on all three receptor

types and TETS is a poor inhibitor of $(\beta_3)_5$ (33, 34). Considering these relationships, we propose that the type B compact set including TETS and TBPS undergoes significant polar interactions in the 1'2' ring, whereas the type A elongated compounds such as EBOB do not. Interestingly, the insecticidal activity of the isoxazoline fluralaner (**10**) seems to result from action at a distinct GABA receptor site (41, 42) not considered here.

TETS Candidate Antidotes. TETS was the first and because of many poisoning cases is now the best known of the small-cage convulsants, but some bicyclophosphorus compounds are much more toxic and probably act in the same way (19, 25). After a half century of search, there are still no adequate antidotes for TETS-induced poisoning, either accidental or intentional. The candidates have come from anticonvulsants used to counteract convulsant action, trials in rats and mice, and mechanism studies in animals, cells, and in vitro systems (*SI Appendix, section S1*). Cell and nerve studies confirm action on GABA-induced signals and chloride flux. Diazepam and Na phenobarbital increase the mouse i.p. LD₅₀ of TETS by severalfold (19) and diazepam and midazolam inhibit [³H]EBOB and [³⁵S]TBPS binding (*SI Appendix, sections S3 and S4*). The highest inhibitory effect among the benzodiazepines and barbiturates examined at 1 or 10 μ M was 30–40% for midazolam and flurazepam (*SI Appendix, section S3*). Several GABA_AR modulators that alter [³⁵S]TBPS binding (30) are also allosteric inhibitors of [¹⁴C]TETS or [³H]EBOB binding. Allopregnanolone is known to be active in [³⁵S]TBPS binding assays (30) and alleviating TETS toxicity (11, 27, 28). NaDMPS, a chelating agent normally used for treating heavy metal poisoning, is effective as a TETS antidote in rodent models and human poisonings proposed to be due to elevating GABA levels rather than as a chelator (*SI Appendix, section S1*). GABA levels are elevated by TETS poisoning in rats and GABA administration relieves the convulsions (*SI Appendix, section S1*). The seizures induced by acute and repeated exposure to TETS are characterized as actions at both GABA and NMDA receptors (28, 29). TETS inhibition of NMDA-induced Ca²⁺ signaling in cultured hippocampal neurons is partially reversed by either, or both, NaDMPS and allopregnanolone (28). Binding of [¹⁴C]TETS or [³H]EBOB, or both, is inhibited by avermectin at 1 μ M and by preganalone, isoguvacine, NMDA, propofol, and pyridoxine but not by NaDMPS at 1 or 10 μ M, whereas bicuculline at 1 μ M stimulated [¹⁴C]TETS and [³H]EBOB binding by 59–68% (*SI Appendix, section S3*). Baclofen at 1 μ M and ethanol at 300 mM had apparently somewhat different effects with the two radioligands (*SI Appendix, section S3*). However, TETS poisoning cases in humans have been treated with diazepam, allopregnanolone, and NaDMPS with little or no benefit (9–15).

The GABA_AR is the target of many toxicants for mammals (TETS) and insects (insecticides) and exists in a multiplicity of subunit and interface combinations (43, 44), allowing high toxicity that reaches its extreme for mammals with TETS and some other small-cage convulsants. In the search for antidotes the GABA_AR in vitro assays described here may provide a rapid means of limiting the number of compounds for animal experimentation and ultimate testing in cases of human poisoning. Further test of this hypothesis requires a larger dataset for inhibition of [¹⁴C]TETS and [³H]EBOB binding versus toxicity.

Materials and Methods

Chemicals and Chromatography. H¹⁴CHO (1 mCi/mL, 250 μ Ci) was purchased from American Radiolabeled Chemicals Inc. H¹³CHO (99 atom % ¹³C, 20% aqueous solution) was obtained from Sigma-Aldrich. [³H]EBOB (26 Ci/mmol) was from Perkin-Elmer Inc.. All other reagents and solvents were obtained from commercial suppliers and used without further purification. The synthesized products were characterized by TLC comparisons on Merck silica gel 60 F₂₅₄ plates detected for unlabeled and [¹³C]TETS by potassium permanganate and for [¹⁴C]TETS by radio TLC using a Bioscan System 200 Imaging Scanner. Purification involved column chromatography using Spe-ed SPE

Cartridges (Super Spe-ed silica gel, 5101; Applied Separations). Radioactivity was determined by liquid scintillation analysis using a Tri-Carb 2810 TR. GC-MS data were recorded on a HP 6890 GC with the 5973 MS instrument.

GABA_AR Membrane Preparation. The preparation method was modified from that of Squires et al. (30). Whole rat brains from Pel-Freez Biologicals stored at -80°C were thawed and homogenized in 50 volumes of ice-cold 1 mM EDTA using a Brinkmann Polytron Homogenizer. The homogenate was centrifuged at $1,000 \times g$ for 10 min, and the supernatant was then centrifuged at $25,000 \times g$ for 30 min. The resulting pellets were suspended in 50 volumes of 1 mM EDTA, packed into cellophane tubing, and dialyzed against distilled/deionized water in an ice-bath (1–2 L, three times for 2 h). The dialyzed suspension was then centrifuged at $25,000 \times g$ for 30 min and the pellets were stored at -80°C .

Binding Assays. The rat brain membrane pellets from storage at -80°C were suspended in ice-cold buffer B [10 mM phosphate buffer (pH 7.5) containing 300 mM NaCl]. Incubation mixtures consisted of membranes (125 μg protein) (45) and 0.5 nM [^3H]EBOB or 1.5 nM [^{14}C]TETS in 1.0 mL of buffer B. After incubation with shaking for 90 min at 37°C , the mixtures were filtered through GF/C filters and rapidly rinsed three times with 5 mL of cold buffer B using a Brandel M-24 cell harvester. Tritium from bound [^3H]EBOB was quantitated by liquid scintillation counting (31). Radiocarbon from [^{14}C]TETS was analyzed by AMS. The filter papers were collected, put in Eppendorf tubes, and held up to 4 wk at 4°C . Then, each filter loaded with protein was placed with 1 μL tributyrin carbon carrier in a quartz tube ($\sim 6 \times 30$ mm, 4 mm i.d.) nested inside two borosilicate glass culture tubes (10×75 mm in 12×100 mm) and dried overnight in a vacuum centrifuge. An excess of CuO (~ 40 mg) was added and the inner quartz vials were transferred to quartz combustion tubes, evacuated, and sealed with a torch. The samples were combusted at 900°C for 3.5 h to oxidize all organic carbon to CO_2 and then reduced to filamentous carbon as previously described (46). Carbon samples were packed into aluminum sample holders, and carbon isotope ratios

were measured on the compact 1-MV AMS spectrometer at the Lawrence Livermore National Laboratory. Typical AMS measurement times were 3–5 min per sample, with a counting precision of 0.6–1.4% and a SD among 3–10 measurements of 1–3%. The $^{14}\text{C}/^{13}\text{C}$ ratios of the protein samples were normalized to measurements of four identically prepared standards of known isotope concentration (IAEA C-6, also known as ANU sucrose) and converted to units of femtograms TETS per microgram protein (47). Each experiment was performed in triplicate and repeated three times in determining the mean and SEs. Curve fitting used the nonlinear (Fig. 3) or linear (Fig. 4) regression program with Prism Software Version 5.0 (GraphPad Software Inc.).

Modeling the GABA_AR Binding Sites. The GABA_AR $\alpha_1\beta_2\gamma_2$ homology model was built with a GluCl template (PDB ID code 3RHW) (48) using previously published protocols (36, 37). Small molecules were parameterized with the PRODRG server (49) and docked into the pore using VinaLC (50). The protein–ligand system was embedded in a lipid bilayer and solvated. Atomistic simulations were performed using GROMACS (51). For more details, see *SI Appendix, section S5*.

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