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Modulation of the $GABA_A$ receptor has been studied with noncompetitive convulsant ligands such as *tert*-butylbicyclophosphorothionate (TBPS) and picrotoxin (PTX). EBOB is a more recently developed ligand that appears to bind in the same region of the channel as TBPS, but with a higher affinity. While only a few studies have examined the binding of EBOB to vertebrate brain tissue and insect preparations, none have examined potential subunit-dependent binding of EBOB. We have thus examined [^{3}H] EBOB binding in rat cerebellum and HEK293 cells stably expressing human $\alpha1\beta2\gamma2$, human $\alpha2\beta2\gamma2$, and rat $\alpha6\beta2\gamma2$ $GABA_A$ receptors. For comparison, [^{35}S] TBPS binding was also examined in $\alpha1\beta2\gamma2$ receptors. Saturation and Scatchard analyses revealed saturable [^{3}H] EBOB binding at one site in all tissue preparations with K_d values ranging from 3 to 9 nM. [^{3}H] EBOB binding, like [^{35}S] TBPS binding, was inhibited by the CNS convulsants dieldrin, lindane, *tert*-butylbicycloorthobenzoate (TBOB), PTX, TBPS, and pentylenetetrazole (PTZ) at one site in a concentration dependent fashion. Affinities were in the high nM to low μM range for all compounds except PTZ (low mM range). GABA modulated [^{3}H] EBOB binding in a biphasic manner in $\alpha1\beta2\gamma2$ receptors with a 100-fold difference between stimulatory and inhibitory affinities. Inhibition of GABA-mediated current by TBOB in $\alpha1\beta2\gamma2$ receptors resulted in a functional IC_{50} of 0.2 μM, in agreement with binding study results. Differences seen in binding between the different receptor subtypes examined suggest that some characteristics of EBOB binding are subunit dependent. In addition, we have shown that [^{3}H] EBOB is a useful ligand in the study of recombinant $GABA_A$ receptors and that results obtained with [^{3}H] EBOB are comparable to those obtained with [^{35}S] TBPS.

[^{3}H] ETHYNYLBICYCLOORTHOBENZOATE ([^{3}H] EBOB) BINDING IN NATIVE AND RECOMBINANT $GABA_A$ RECEPTORS

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THESIS

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TABLE OF CONTENTS

LIST OF TABLES

Table Page

LIST OF ILLUSTRATIONS

Figure Page

CHAPTER I. INTRODUCTION

$GABA_A$ Receptor Background

GABA (γ-aminobutyric acid), a small amino acid, is the major inhibitory neurotransmitter in the central nervous system (CNS) and has receptors consisting of three different types: $GABA_A$, $GABA_B$, and $GABA_C$. GABA is formed from glutamate by the enzyme glutamic acid decarboxylase (Hevers and Luddens, 1998). GABA is catabolized by first being actively transported into neurons and glia and then converted to succinic semialdehyde by GABA transaminase (Taylor and Insel, 1990). $GABA_A$ receptors are hetero-oligomeric fast ligand-gated chloride channels that span the cell membrane (DeLorey and Olsen, 1992). $GABA_C$ receptors, most prominently expressed in the retina, are either homo-oligomeric and composed solely of ρ subunits or hetero-oligomeric and composed of ρ and $GABA_A$ receptor subunits (Cutting et al., 1991; Enz and Cutting, 1998). These receptors show unusual pharmacological properties such as insensitivity to such drugs as barbiturates, benzodiazepines, and bicuculline (Smith and Olsen, 1995; Hevers and Luddens, 1998). $GABA_B$ receptors are G-protein coupled receptors linked to K^+ or Ca^{++} channels (MacDonald and Olsen, 1994). The $GABA_A$ receptor belongs to the ligand-gated ion channel superfamily, which also includes the nicotinic acetylcholine receptor, glycine receptor, and 5-hydroxytryptamine$_3$ ($5\text{-}HT_3$) receptor (DeLorey and Olsen, 1992; Barnes, 1996; Rabow et al., 1995; Smith and Olsen, 1995).

The $GABA_A$ receptor chloride channel complex is believed to consist of five subunits, with each subunit having four putative hydrophobic membrane-spanning domains (TM1-TM4), with the TM2 domain most likely contributing to the wall of the channel (DeLorey and Olsen, 1992; Rabow et al., 1995; Smith and Olsen, 1995). The large intracellular loop between TM3 and

TM4 contains consensus sequences for phosphorylation by various kinases (Rabow et al., 1995; Hevers and Luddens, 1998). Each subunit possesses a large extracellular amino (NH_2) domain that likely includes the ligand binding site and a small extracellular carboxy (COOH) domain (Rabow et al., 1995; Hevers and Luddens, 1998). $GABA_A$ receptors are the primary transducers of fast inhibitory transmission in the vertebrate CNS, are ubiquitously present in the mammalian brain, and are modulated by compounds such as benzodiazepines, barbiturates, neurosteroids, anesthetics, polyvalent cations, ethanol, penicillin (at high concentrations), and convulsant agents (Barnes, 1996; Rabow et al., 1995; Macdonald and Olsen, 1994; Stephenson, 1995; Hevers and Luddens, 1998). The binding of GABA to this receptor opens the chloride channel and causes an influx of chloride ions down their concentration gradient, which evokes a hyperpolarizing current (Hevers and Luddens, 1998). Drugs such as barbiturates, benzodiazepines, and anesthetics enhance this inhibitory current, while drugs like picrotoxin (PTX) and other noncompetitive convulsant agents, the competitive antagonist bicuculline, and the open channel blocker penicillin, diminish or abolish this current (Macdonald and Olsen, 1994). Major binding domains of the $GABA_A$ receptor include sites for GABA, benzodiazepines, barbiturates, picrotoxin, and anesthetic steroids (DeLorey and Olsen, 1992; Hevers and Luddens, 1998). Figure 1 shows two representations of the $GABA_A$ receptor with its 5 membrane spanning subunits and various binding sites. Various subunit subtypes of $GABA_A$ receptors have been discovered in mammals (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, and ρ_{1-3}), and various isoforms of these subunits exist that most likely combine in a pentameric structure to form numerous subtypes (Barnes, 1996; Rabow et al., 1995; Smith and Olsen, 1995; Stephenson, 1995; Hevers and Luddens, 1998).

The distribution of $GABA_A$ receptor subtypes in the brain is ubiquitous, but at the same time, region specific. For example, subtype compositions that include $\alpha 1$ and $\gamma 2$, especially $\alpha 1\beta 2\gamma 2$, are present in nearly all brain regions and are the most abundant receptor subtype

(Rabow et al., 1995; Hevers and Luddens, 1998). The expression of other subunits, such as β1 or α6, are restricted to the hippocampus and cerebellar cortex, respectively (Rabow et al., 1995). In addition, cerebellar cells (most notably the granule cell layer) express most abundantly the following combinations of subunit mRNA: α1, α6, β2, β3, γ2, and δ (Laurie et al., 1992; Wisden et al., 1996; Korpi et al., 1996). The following subunit mRNAs have been detected in the following regions of rat cerebellum to significant levels: stellate/basket cells (molecular layer) α1, β2, γ2; purkinje cells α1, β2, β3, γ2; granule cells α1, α6, β2, β3, γ2, δ; and Bergmann glia α2, γ1 (Laurie et al., 1992; Wisden et al., 1992). The cerebellar granule cell layer is the only site expressing α6 mRNA in the entire CNS, and the two types of receptors formed are most likely α6βxγ2 and α1/6βxδ (Laurie et al., 1992). The δ subunit is likely colocalized with α4 and α6 subunits, and its expression in the cerebellum is dependent on the expression of the α6 subunit (Wisden et al., 1991; Jones et al., 1997).

Distinct pharmacological differences are found between insect and vertebrate $GABA_A$ receptors (Sattelle et al., 1991). For example, from functional and binding studies, bicuculline is ineffective, some steroids are weakly effective, some convulsants and cyclodienes are moderately effective, and some depressants such as pentobarbital are weakly to moderately effective in insect receptors compared to vertebrate receptors (Sattelle et al., 1991). *tert*-Butylbicycloorthobenzoate (TBOB) and *tert*-butylbicyclophosphorothionate (TBPS) have higher affinities in vertebrate compared to insect receptors (Sattelle et al., 1991).

The Convulsant Binding Site

The modulation of the $GABA_A$ receptor by various compounds is subunit dependent and has been studied with uncompetitive convulsant ligands such as TBPS and PTX (Obata and Yamamura, 1988). For example, the GABA binding site depends on both the α and β subunits (reviewed by Hevers and Luddens, 1998). Benzodiazepine modulation is dependent on both α and

γ subunit types (McKernan et al, 1995; reviewed by Hevers and Luddens, 1998). Compounds believed to bind inside the channel of the $GABA_A$ receptor include the bicyclo "cage" compounds TBOB and TBPS, the plant toxin picrotoxin, the polycyclic convulsant pentylenetetrazole (PTZ), and the polychlorocycloalkane insecticides dieldrin (a cyclodiene) and lindane (hexachloro-cyclohexane, γ isomer) (Macdonald and Olsen, 1994; Obata et al., 1988; Huang and Casida, 1996; Casida and Palmer, 1988). This region, known as the noncompetitive convulsant binding site, is distinct from the GABA binding site and when occupied, inhibits GABA-induced hyperpolarization. The classical drug used to study the noncompetitive convulsant site is picrotoxin, the natural plant toxin that is a mixture of the active component picrotoxinin and the inactive component picrotin (Casida, 1993; Macdonald and Olsen, 1994). PTX likely inhibits GABA-mediated current not by directly blocking the channel, but most likely by allosterically affecting the conformational change that leads to the opening of the chloride channel once GABA binds, thus possibly altering the intrinsic gating of the channel (Twyman et al., 1989).

TBPS, another member of the trioxabicyclooctane class in addition to TBOB and EBOB, is a bicyclophosphorothionate that belongs to the bicyclophosphorus esters, was developed as a radioligand for the GABA-gated chloride ion channel, and like TBOB, is more toxic to mammals than to insects (Casida and Palmer, 1988; Casida, 1993). TBOB and EBOB belong to the bicycloorthobenzoates (bicycloorthocarboxylates) and were developed from the bicyclic esters by optimizing substituents for potency and selective toxicity (Casida, 1993). The terminal regions of bicycloorthocarboxylates are critical in determining the inhibitory potency of competing [^{35}S] TBPS binding (Casida et al., 1985). In one study, the inhibition of GABA-stimulated $^{36}Cl^-$ uptake was strongly dependent upon substituents at positions 1 and 4 of bicycloorthocarboxylates and bicyclophosphorus esters (Obata et al., 1988). Position 4 is where the t-butyl resides in TBOB and TBPS, while position 1 is on the opposite end of the molecule, for example, where P=S in TBPS.

The chemical formula of the trioxabicyclooctanes is $RC(CH_2O)_3X$, where R represents t-butyl, and X represents P=S, in TBPS (Casida et al., 1985). From structure-activity relationships, high potency $GABA_A$ receptor trioxabicyclooctane noncompetitive antagonists have as an optimal 4-substituent a hydrophobic group, and as an optimal 1-substituent, a phenyl (Ph) moiety with one or more electron withdrawing groups (Casida et al., 1985). Thus, compounds can be designed with increased convulsant activity and potency by varying the substituents at the 1- and 4-positions, an indication that each terminal region of the molecule is critical in binding (Casida et al., 1985).

[^{35}S] TBPS and [^{3}H] TBOB, developed as radioligands for the convulsant binding site, have replaced the original radioligand ligand used to study this site, [^{3}H] dihydropicrotoxinin ([^{3}H] DHP). [^{3}H] DHP binds to rat brain homogenates with low specific binding and an affinity of 1-2 μM, a poor affinity for a radioligand, and is no longer marketed (Casida and Palmer, 1988; Ticku, et al., 1978; Cole and Casida, 1992). [^{35}S] TBPS has proven to be successful and is the prototypical radioligand used in binding studies of the noncompetitive convulsant site and to investigate allosteric coupling with other sites on the $GABA_A$ receptor (Ramanjaneyulu and Ticku, 1984; Slaney et al., 1995; Korpi and Luddens, 1993; Zezula et al., 1996; Luddens et al., 1994; Peris et al., 1991; Im et al., 1994; Davies et al., 1997). Ethynylbicycloorthobenzoate (EBOB), a recently developed ligand that appears to bind to this same site or an overlapping site, possesses a few advantages over TBPS, the conventional radiolabeled ligand currently used in $GABA_A$ binding studies (Hawkinson and Casida, 1992; Huang and Casida, 1996; Cole and Casida, 1992; Squires et al., 1983; Pregenzer et al., 1993; Korpi and Luddens, 1993). EBOB typically has at least a 10-fold higher affinity than TBPS for this binding site, a longer half-life (EBOB is labeled with ^{3}H and TBPS with ^{35}S, the difference in half-life being 12 years vs. 87 days), and also costs less. Disadvantages are discussed in the discussion section.

Even though [^{3}H] TBOB and [^{35}S] TBPS have improved specific binding and higher affinities relative to [^{3}H] DHP binding and are used in mammalian brain with good results, they have low insecticidal activity and cannot be used to adequately measure binding properties at the noncompetitive convulsant site in insect preparations (Cole and Casida, 1992). EBOB has proven to be an improved radioligand with higher affinity for the noncompetitive convulsant binding site in vertebrate and insect preparations (Cole and Casida, 1992) and in quantitative autoradiography studies of both rat and human brain (Kume and Albin, 1994). Studies examining [^{3}H] EBOB binding in only vertebrate brain tissue and insects have been published (Hawkinson and Casida, 1992; Huang and Casida, 1996; Cole and Casida, 1992; Kume and Albin, 1994). Only one study has been published on [^{3}H] TBOB binding in a specific $GABA_A$ receptor subtype (Pericic et al., 1998). To the best of our knowledge, no literature is available on [^{3}H] EBOB binding in recombinant $GABA_A$ receptor subtypes.

Problem/Hypothesis and Significance

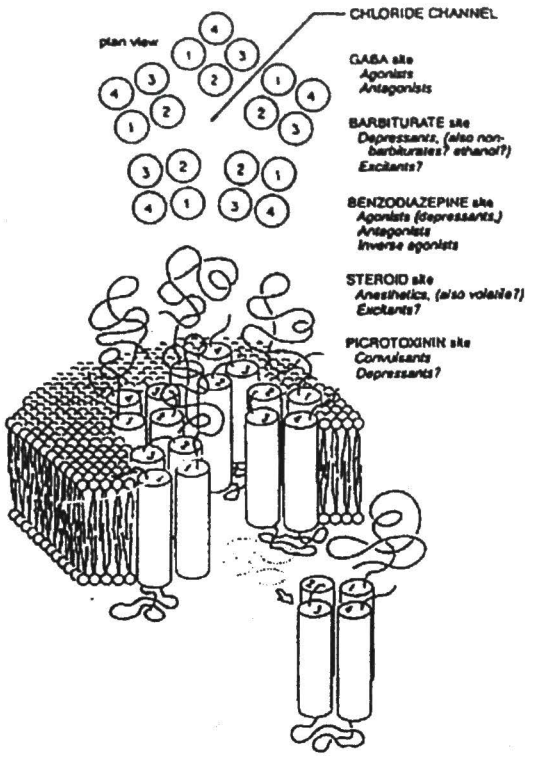
TBPS has proven to be useful in studying the pharmacology of the $GABA_A$ receptor. Published results on EBOB suggest that it may also be useful in analysis of the convulsant binding site. However, no binding studies have been performed with EBOB in recombinant receptors to investigate pharmacological differences of this site between different receptor subtypes or allosteric coupling to this site by compounds binding to other sites on the receptor. We thus wanted to investigate EBOB's utility as a radioligand to investigate the noncompetitive convulsant site and allosteric sites, and if it proves to be at least as useful as TBPS. In addition, a major goal of our laboratory is to study the convulsant binding site and to determine the critical determinants of this site. Based on our results, EBOB is proving to be a useful and superior tool to TBPS in the study of the pharmacology of $GABA_A$ receptors and also shows subunit dependent binding.

One of our goals was to access differences between native tissue and specific receptor subtypes. The characteristics of [^{3}H] EBOB binding and interaction with various convulsant compounds in native tissue and recombinant receptors are compared to published data on [^{35}S] TBPS and [^{3}H] TBOB. We report the interactions of [^{3}H] EBOB with various convulsant compounds in rat cerebellum, human $\alpha1\beta2\gamma2$, human $\alpha2\beta2\gamma2$, and rat $\alpha6\beta2\gamma2$ $GABA_A$ receptor subtypes along with the difference in affinity of these four different tissue types for [^{3}H] EBOB. The allosteric effect of GABA on [^{3}H] EBOB binding is examined along with TBOB's (substituted for EBOB because of structural similarity and unavailability of unlabelled EBOB) functional inhibition of GABA-mediated chloride current in human $\alpha1\beta2\gamma2$ receptors. Any similarities or differences are noted so that a better understanding of subunit-dependent binding of convulsant compounds can be obtained. In addition, the justification of using [^{3}H] EBOB as a radioligand to study the noncompetitive convulsant site in recombinant $GABA_A$ receptors is addressed.

Because GABA is the major inhibitory neurotransmitter in the CNS, its receptors are the sites of many therapeutics. TBPS has been used to investigate the coupling of various therapeutic drugs to the noncompetitive convulsant binding site. We wanted to begin to address the utility of [^{3}H] EBOB in the investigation of allosteric compounds, including therapeutics. Because multiple subunit combinations of $GABA_A$ receptors exist in the brain, understanding the different subunit-dependence of convulsant drug binding and functional receptor activation may ultimately lead to a better understanding of which brain regions are more susceptible to convulsant activity based on receptor subtype distribution. This can lead to the development of more selective anticonvulsant or anti-epileptic drugs that target desired receptor subtypes without the production of unwanted side effects due to the targeting of other receptor subtypes. Studying how the convulsant site couples to other sites targeted by therapeutics can also lead to the development of

more selective therapeutic compounds used as antianxiety, sedative/hypnotic, muscle relaxant, or anesthetic agents. This difference in subunit dependent binding of convulsant drugs may also be useful in identifying receptor subtype presence in various brain regions and in understanding the structural differences of the noncompetitive convulsant binding site between different receptor subtypes. In addition, [^{3}H] EBOB is a radioligand of improved affinity that can be used in both insect and vertebrate preparations. Insecticide action can be better understood in terms of species specificity (Cole and Casida, 1992), something not appropriate with [^{3}H] TBOB and [^{35}S] TBPS because of their low insecticidal activity.

A.



B.

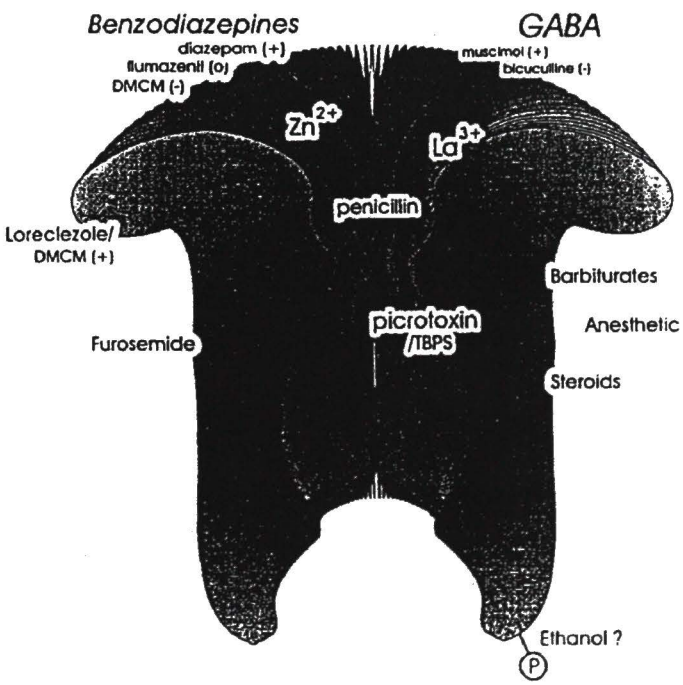
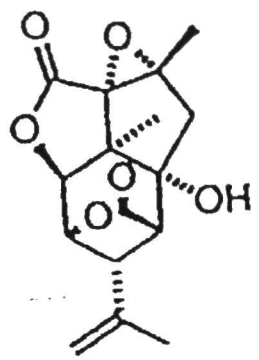
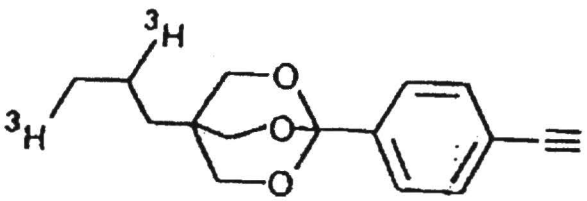


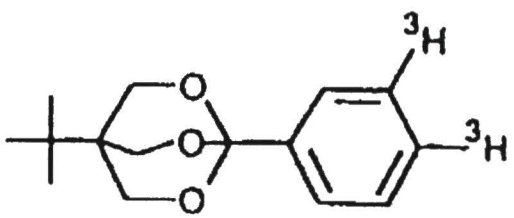
Figure 1. Model of the $GABA_A$ Receptor Structure. Panel A. (Tyndale et al., 1995). Panel B. (Hevers and Luddens, 1998).



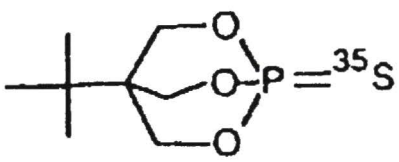
Picrotoxin



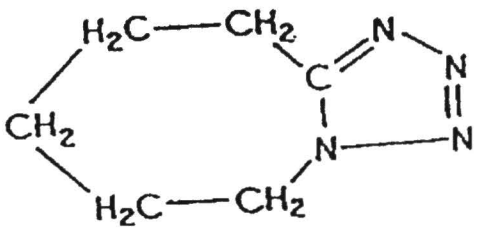
[^{3}H] EBOB



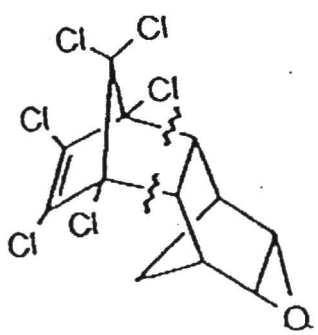
[^{3}H] TBOB



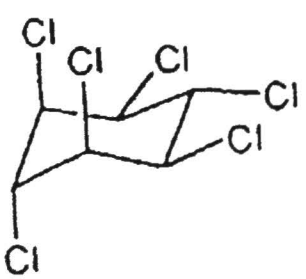
[^{35}S] TBPS



PTZ



Dieldrin



Lindane

Figure 2. Structures of the Convulsant Compounds.

CHAPTER II. METHODS

Chemicals

[^{3}H] EBOB (38.0 and 30.00 Ci/mmol) and [^{35}S] TBPS (102.7 – 122.5 Ci/mmol) were purchased from New England Nuclear (NEN). TBOB was provided as a gift from Doug Covey (Washington University, St. Louis, MO). GABA, dieldrin, lindane (hexachlorocyclohexane, γ isomer), PTX, PTZ, and bicuculline methiodide were obtained from Sigma. TBPS was purchased from Research Biochemical International (RBI). All stocks excluding PTZ and GABA were made in dimethylsulfoxide (DMSO) and diluted in buffer (200 mM NaCl, 25 mM HEPES, pH 7.4 or 200 mM NaCl, 50 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4) so that the final DMSO concentration was < 0.2% in cerebellar assays and < 0.4% in recombinant assays. PTZ and GABA stocks were made in buffer (200 mM NaCl, 25 mM HEPES, pH 7.4 or 200 mM NaCl, 50 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4).

HEPES and $NaH_2PO_4 \cdot 2H_2O$ were purchased from Fisher Scientific. DMSO, NaCl, CsCl, EGTA, Mg-ATP, KCl, $MgCl_2$, $CaCl_2$, D-glucose, and fetal bovine serum were purchased from Sigma. EDTA was purchased from Stratagene. Minimum Essential Medium Eagle (MEM) and G418 sulfate were purchased from Cellgro. L-glutamine, penicillin/streptomycin, MEM sodium pyruvate, trypsin, EDTA, and Dulbecco's Modified Eagle Medium (DMEM) were purchased from GibcoBRL. Hygromycin was purchased from Calbiochem. Ciprofloxacin was purchased from Bayer. The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce.

Cloned $GABA_A$ Receptors

HEK293 (human embryonic kidney) cells stably expressing configurations of rat and human $GABA_A$ receptors were generously supplied by Don Carter of Pharmacia-Upjohn (Kalamazoo, MI) and John Drewe of Cytovia (San Diego, CA). A detailed description of the

preparation of HEK293 cells stably expressing $GABA_A$ receptors has been published previously (Hamilton et al., 1993). HEK293 cells stably expressing rat $\alpha1\beta2\gamma2S$, $\alpha6\beta2\gamma2S$, $\beta2\gamma2S$, and human $\alpha1\beta2\gamma2S$ and $\alpha2\beta2\gamma2S$ configurations of the receptor were examined.

Tissue Preparation

Rat Cerebellum

Homogenized rat cerebellum (from adult Long Evans male hooded rats) in Krebs-Ringers HEPES (KRH) buffer (14 mM NaCl, 0.15 mM KCl, 0.2 mM $CaCl_2$, 0.1 mM $MgSO_4$, and 2.5 mM HEPES) was generously given to us by Dr. Christopher and NancyEllen de Fiebre. The homogenate was first centrifuged at 40,000g. The pellet was washed by resuspension in homogenization buffer (10 mM HEPES, 1 mM EDTA, pH 7.4), homogenized with a Brinkmann polytron (speed 6 for 10-20 sec), and recentrifuged. This process was repeated. The pellet was then resuspended in buffer to give a final protein concentration of 2-3 μg/μl as determined by the BCA protein assay method and then stored at -80°C.

Growth and Preparation of HEK Cells

Recombinant HEK293 cells expressing human $\alpha1\beta2\gamma2$ and human $\alpha2\beta2\gamma2$ $GABA_A$ receptors were grown in DMEM supplemented with 10% fetal calf serum, L-glutamine, penicillin/streptomycin, sodium pyruvate, hygromycin, and ciprofloxacin. Cells expressing rat $\alpha1\beta2\gamma2$, rat $\alpha6\beta2\gamma2$, and rat $\beta2\gamma2$ $GABA_A$ receptors were grown in MEM supplemented with 10% fetal calf serum, L-glutamine, penicillin/streptomycin, G418, and ciprofloxacin. Untransfected HEK cells were grown in MEM supplemented with 10% fetal calf serum, L-glutamine, penicillin/streptomycin, and ciprofloxacin. All cells were initially grown in T-75 flasks (Falcon), then seeded onto 150 mm plates (Corning), and harvested when 90-100% confluency was reached. Old media was discarded, fresh media was added to each plate, cells were scraped gently and then placed in test tubes over ice. Whole cells were centrifuged at

2000g. The pellet was washed twice by resuspending in sodium phosphate buffer (200 mM NaCl, 50 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4) and then recentrifuging. The supernatant was discarded and the pellet was homogenized by resuspending in buffer and polytroning at speed 6 for 10-20 sec. The homogenate was then centrifuged at 40,000g. The supernatant was discarded and buffer was added to the pellet to result in a protein concentration of 2-7 μg/μl as determined by the BCA protein assay method. The mixture was polytroned at speed 6 for 30-40 sec and then stored at -80°C.

Binding Assays

Rat Cerebellum

Tissue was thawed the day of the assay and diluted to the desired concentration in 200 mM NaCl, 25 mM HEPES buffer, pH 7.4. For the saturation assays, 50 μg of cerebellar tissue was incubated with varying concentrations of [^{3}H] EBOB and 50 μM of the GABA competitive antagonist bicuculline methiodide in a final volume of 150 μL (200 mM NaCl, 25 mM HEPES buffer, pH 7.4) for 1 hour at 37°C. Bicuculline, a competitive antagonist at the GABA binding site, was used to increase [^{3}H] EBOB binding sites in the cerebellum. [^{3}H] EBOB binding sites are allosterically blocked by endogenous GABA in certain layers of the cerebellum, such as the granule cell layer (Korpi et al., 1992; Korpi and Luddens, 1993). For competition assays, 50 μg of tissue was incubated with 3-4 nM [^{3}H] EBOB (fixed concentration in each experiment), 50 μM bicuculline methiodide, and varying concentrations of convulsant compound (dieldrin, lindane, PTX, TBPS, or PTZ) in a final volume of 150 μL (200 mM NaCl, 25 mM HEPES buffer, pH 7.4) for 1 hour at 37°C. For both saturation and competition experiments, 100 μM PTX was used to define nonspecific binding. In competition experiments, nonspecific binding was approximately 20% of total binding. All assays were performed in 96 well polyvinyl chloride (PVC) flexible assay plates (Falcon #3912, Microtest III). In all saturation and competition experiments, bound

ligand was separated from free on glass fiber filters (Gelman Sciences, Type A/E and Advantec MFS, GB100R) using an Inotech cell harvester. Filters were rinsed with approximately 300 µl (well capacity) of wash buffer (200 mM NaCl, 6.25 mM HEPES, pH 7.4) four times.

Recombinant HEK Cells

Tissue was thawed the day of the assay, diluted in phosphate buffered saline (PBS, 200 mM NaCl. 50 mM $NaH_2PO_4 \cdot 2H_2O$, pH 7.4) to the desired concentration, and polytroned at speed 6 for 30-40 seconds. For saturation assays, a fixed amount of recombinant tissue (50-300 µg) was incubated with varying concentrations of [^{3}H] EBOB in PBS to a final volume of 500 µL for 90 minutes at room temperature. Non-specific binding was defined with 100 µM or 31.6 µM TBPS. For competition assays with [^{3}H] EBOB, a fixed tissue amount (100-200 µg) was combined with 2-5 nM [^{3}H] EBOB (fixed concentration in each experiment) and varying concentrations of convulsant compound or GABA (human $\alpha1\beta2\gamma2$ experiments only) in PBS to a final volume of 500 µL. Non-specific binding was defined with 100 µM TBPS or 10 µM TBOB. Samples were incubated for 90 minutes at room temperature in 48-well siliconized polystyrene tissue culture plates (Falcon #3078) or in borosilicate glass tubes (Fisher Scientific). For [^{35}S] TBPS binding, 90 or 150 µg of human $\alpha1\beta2\gamma2$ tissue was combined with 2.3 or 2.6 nM of [^{35}S] TBPS and varying concentrations of convulsant compound in PBS to a final volume of 500 µL. 10 µM TBPS was used to define nonspecific binding. Samples were incubated for 90 minutes at room temperature. For all recombinant cell competition assays, bound ligand was separated from free on glass fiber filters using either an Inotech cell harvester or a Millipore 12-well manifold vacuum filtration system. For all saturation assays, the Inotech cell harvester was used for filtration. In competition experiments with human $\alpha1\beta2\gamma2$ and $\alpha2\beta2\gamma2$ tissue filtered with the Millipore manifold, nonspecific binding was roughly 40-50% of total binding. When the Inotech cell harvester was used with these receptor types, nonspecific binding was roughly 10-15% of

total binding. Even though the Inotech cell harvester was the only vacuum filtration device used with rat α6β2γ2 tissue, nonspecific binding was very high and 60-70% of total binding in competition experiments. For the Millipore vacuum, the "on reaction" was terminated by adding 5 ml of PBS. The contents were immediately filtered over glass fiber filters (Schleicher & Schuell #30, 2.5 cm). Each filter was rinsed twice with 5 ml of buffer. For the Inotech cell harvester, each sample was filtered over 2 layers of glass fiber filters (Gelman Sciences, Type A/E and Advantec MFS, GB100R) and then rinsed 3 times with approximately 1.5 ml (well capacity) of buffer.

Problems were experienced with [^{3}H] EBOB apparently adhering completely to polystyrene and partially to polypropylene. To solve this problem, plasticware containing [^{3}H] EBOB made in buffer or incubation mixtures with [^{3}H] EBOB, such as the 48-well polystyrene plates, was siliconized. Plates were siliconized with dichlorodimethylsilane, as explained by Sambrook et al. (1989) in detail.

Experimental Corrections

Some difficulties were encountered when performing binding experiments with dieldrin. In cells transfected with recombinant $GABA_A$ receptors, dieldrin resulted in dose-dependent inhibition at lower concentrations, but paradoxal stimulation of [^{3}H] EBOB binding at higher concentrations. This stimulation was also observed in untransfected HEK cells and other HEK cells expressing other receptor subtypes (rat α1β2γ2 and rat β2γ2) in which no specific binding was achieved (data not shown). Thus, the stimulation of [^{3}H] EBOB binding observed at high dieldrin concentrations was determined to be nonspecific in nature and the data were corrected for this paradoxal stimulation.

All Binding Assays

Filters were placed in scintillation vials, 3 ml of CytoScint ES liquid scintillation cocktail (ICN Pharmaceuticals) was added, and radioactivity was accessed with a Packard Tri-Carb 2100 TR Liquid Scintillation Analyzer the next day.

Data Analysis for Binding Experiments

Origin 5.0 was used for plotting and fitting linear, hyperbolic, and sigmoidal functions. The following hyperbolic function was used to fit the one-site saturation data: $y = (B_{max} * x)/(K_d + x)$, where y represents specific binding to receptors and x is the radioligand concentration. The saturation data were also transformed algebraically to generate a Scatchard plot and linear regression used to generate the best line through the points. The K_d (dissociation constant, indicates concentration of radioligand at 50% maximal binding), which is a measure of binding affinity, and B_{max} (the total number of receptors bound by radioligand) were determined from saturation and Scatchard analysis and compared. IC_{50} values (the concentration of drug that inhibits 50% of maximal radioligand binding) were obtained by fitting points from the competition experiments with the following logistic function: $y = ((A_1 - A_2)/(1 + (x/IC_{50})^{nH})) + A_2$, where A_1 is the initial y value, A_2 is the final y value, and n_H is the Hill coefficient. The Cheng and Prushoff equation was used to determine K_i, where $K_i = IC_{50}/(1+[L]/K_d)$, where [L] is radioligand concentration (Cheng and Prusoff, 1973). K_i represents the true affinity of the competing drug assuming that no radiolabelled compound is present. Interactive Statistical Programs (ISP), version 4.04, was used to perform one-way Analysis of Variance (ANOVA) followed by the Tukey comparison to obtain statistical differences between K_d, B_{max}, and K_i values.

Electrophysiology: Whole Cell Patch Clamping

Whole-cell patch recordings were made at room temperature (22-25°C). Cells were voltage-clamped at -60 mV. Patch pipettes of borosilicate glass (1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 1-2.5 MΩ for whole-cell recordings. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg-ATP, pH 7.2. Coverslips containing HEK cells expressing human $\alpha 1\beta 2\gamma 2$ receptors were placed in a small chamber (~ 1.5 ml) on the stage of an inverted light microscope (Olympus IMT-2) and superfused continuously (5-8 ml/min) with the following external solution containing (in mM): 125 NaCl, 5.5 KCl, 0.8 $MgCl_2$, 3.0 $CaCl_2$, 20 HEPES, 10 D-glucose, pH 7.3. GABA-induced Cl^- currents were obtained using an Axoclamp 200A amplifier (Axon Instruments, Foster City, CA) equipped with a CV-4 headstage, low-pass filtered at 5 kHz, monitored on a Hitachi digital oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.0, Axon Instruments) for subsequent analysis.

Experimental Protocol

GABA, with or without varying concentrations of TBOB, was prepared in the extracellular solution and was applied from independent reservoirs by gravity flow for 20 seconds to the cells using a Y-shaped tube positioned within 100 μm of the patched cell. Receptors were activated with 10 μM GABA, the approximate EC_{25} in human $\alpha 1\beta 2\gamma 2$ receptors based on experiments in our laboratory, for 20 seconds. This concentration was chosen because minimal desensitization was elicited. The GABA control response was established from two consecutive recordings with the same current amplitude (at least one minute was allowed between each recording so that GABA could wash out, and receptors could recover from desensitization, if present). After the control was established, the effect of TBOB on the response was examined.

Because TBOB was slow to occupy the receptors, as indicated by a lack of or a minimal inhibition of current with initial applications, repeated applications were necessary. TBOB concentrations varying from 0.01 μM to 3 μM were applied for 20 seconds along with 10 μM GABA consecutively a total of 12 times, with a 20 second pause between each application. The same protocol was performed using GABA alone to control for time dependent changes in GABA-induced current. Because most cells did not fully recover from TBOB inhibition, it was necessary to obtain a new patch on a different cell for most data points. Peak and steady state currents were computed for the 1^{st} and 12^{th} applications as a percentage of control. The correction for the average time dependent current change was applied to the results from the 12^{th} application. A dose response curve of the inhibition by TBOB of 10 μM GABA-induced chloride current amplitude (steady state) in human $\alpha 1\beta 2\gamma 2$ receptors was generated with Origin 5.0. The IC_{50} value was determined after the logistic function was used to fit the points (see data analysis of binding experiments section for the equation).

CHAPTER III. RESULTS

[^{3}H] EBOB Saturation Studies in Rat Cerebellum, Human α1β2γ2, Human α2β2γ2, and Rat α6β2γ2 $GABA_A$ Receptors

Saturation analyses were performed in rat cerebellum, human α1β2γ2, human α2β2γ2, and rat α6β2γ2 receptors to determine the K_d and B_{max} of each tissue type for [^{3}H] EBOB. Representative saturation and Scatchard plots are shown in Figure 3 for human α1β2γ2 and rat α6β2γ2 receptors. Each plot is representative of one experiment performed in triplicate. It should be noted from the rat α6β2γ2 saturation plot that at [^{3}H] EBOB concentrations greater than 5 nM, nonspecific binding was greater than specific binding.

Table 1. Summary of [^{3}H] EBOB Saturation Binding Data in Rat Cerebellum, Human α1β2γ2, Human α2β2γ2, and Rat α6β2γ2 $GABA_A$ Receptors

	Rat Cerebellum	Human α1β2γ2	Human α2β2γ2	Rat α6β2γ2
K_d ± s.e.m (nM)	4.43 ± 0.94	8.82 ± 0.57	9.33 ± 0.88	3.04 ± 0.36
B_{max} ± s.e.m (fmol/mg protein)	2035 ± 133	4025 ± 73	3674 ± 181	127 ± 13

Table 1 summarizes the saturation data in all receptor subtypes. The K_d and B_{max} values were the average of 3 or 4 experiments ± the standard error of the mean (S.E.M.). The results obtained from Scatchard analysis agreed with saturation results to within 20% for K_d values and 6% for B_{max} values. [^{3}H] EBOB bound to a single saturable site in all 4 receptor types examined. The criteria used to establish the single site conclusion was that the correlation for all linear fits was $r^2 > 0.92$ in cerebellum, α1β2γ2, and α2β2γ2 receptors and $r^2 > 0.61$ in α6β2γ2 receptors (most of the correlation in α6β2γ2 receptors was near or greater than 0.81). Rat α6β2γ2 receptors had the highest affinity for [^{3}H] EBOB, followed by rat cerebellum, with a comparable

affinity roughly 1.5 fold lower, and lastly, by human α1β2γ2 and α2β2γ2 receptors, both with roughly equal affinities that were 3-fold lower than the rat α6β2γ2 affinity. Human α1β2γ2 and α2β2γ2 receptors were expressed at the highest levels in HEK cells (4000 fmol/mg protein range), followed by a 2 fold lower expression of $GABA_A$ receptors in rat cerebellum, and a 30 fold lower expression of rat α6β2γ2 receptors in HEK cells. The following significant differences were found in K_d and B_{max} values between the different receptor subtypes with the ANOVA test followed by the Tukey comparison.

K_d:	rat cerebellum and human α1β2γ2	for all, $p < 0.01$
	rat cerebellum and human α2β2γ2	
	human α1β2γ2 and rat α6β2γ2	
	human α2β2γ2 and rat α6β2γ2	
B_{max}:	rat cerebellum and human α1β2γ2	for all, $p < 0.01$
	rat cerebellum and human α2β2γ2	
	rat cerebellum and rat α6β2γ2	
	human α1β2γ2 and rat α6β2γ2	
	human α2β2γ2 and rat α6β2γ2	

Not finding any significant differences between the affinities in cerebellum and α6β2γ2 receptors may indicate that the cerebellum likely contains a substantial amount of α6β2γ2 receptors, which are present in the granule cell layer. The results also suggest that the α subunits of α1β2γ2 and α2β2γ2 receptors share similar binding affinities for [^{3}H] EBOB, while the affinity in α6 containing receptors is different.

A significant level of [^{3}H] EBOB specific binding was not observed using preparations of rat α1β2γ2 and rat β2γ2 receptors. This was most likely due to a low receptor expression in both receptor types. Results from rat α1β2γ2 receptors were supposed to be compared to human α1β2γ2 results to see if the species difference would have resulted in different affinities. The effect of the lack of the α subunit on [^{3}H] EBOB binding affinity would have been determined from experiments with β2γ2 receptors.

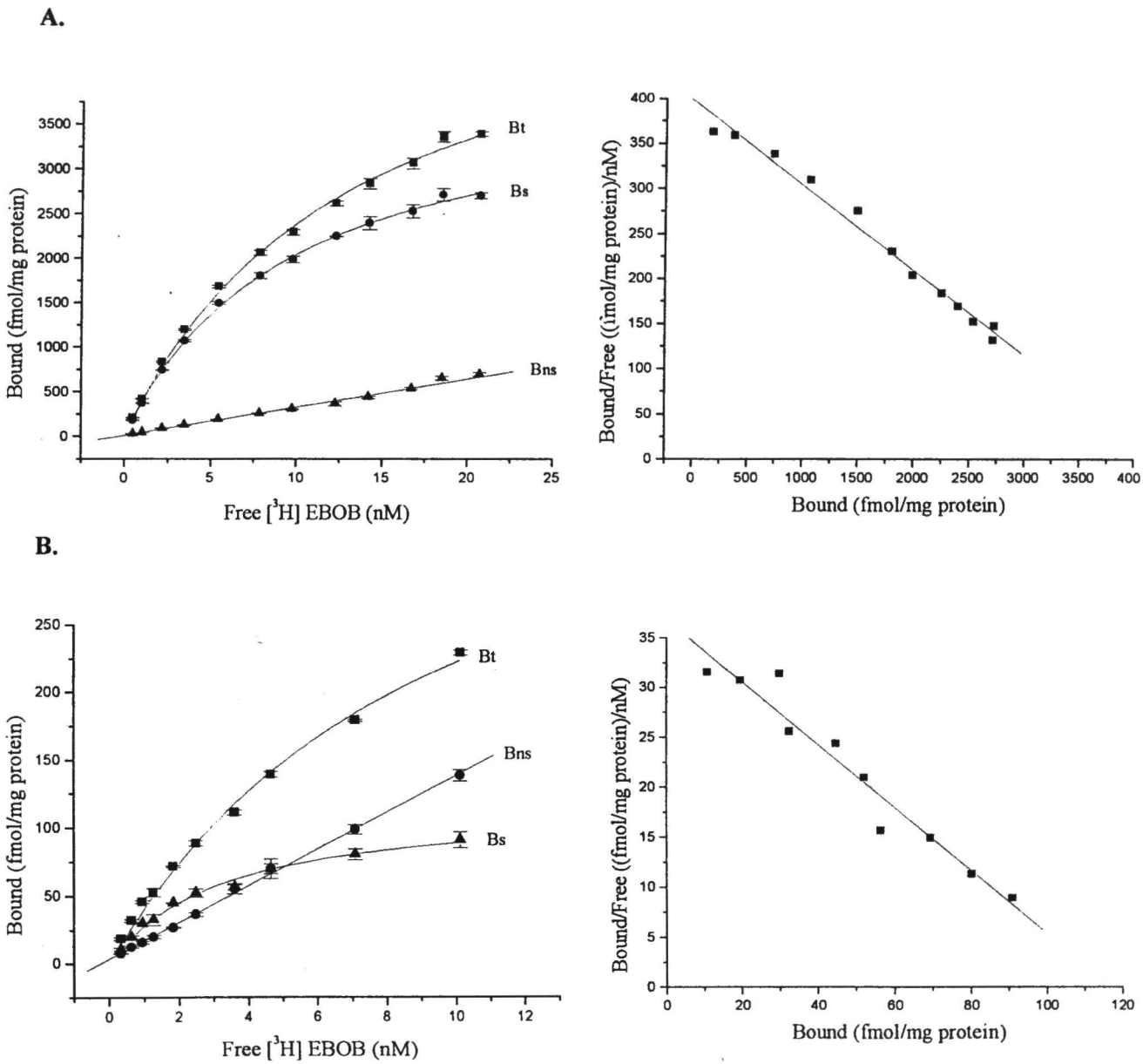


Figure 3. **Saturation and Scatchard Curves of [^{3}H] EBOB Binding in (A) Human α1β2γ2 and (B) Rat α6β2γ2 GABA$_A$ Receptors.** The data were representative of one of 3 or 4 experiments performed in triplicate. K_d = 9 nM in human α1β2γ2 and 3 nM in rat α6β2γ2 receptors. Note the lower expression of rat α6β2γ2 vs. human α1β2γ2 receptors (B_{max} = 127 and 4025 fmol/mg protein, respectively). Bt is total binding, Bns is nonspecific binding, and Bs is specific binding.

Competition of [^{3}H] EBOB Binding by Convulsant Compounds in Rat Cerebellum, Human $\alpha1\beta2\gamma2$, Human $\alpha2\beta2\gamma2$, and Rat $\alpha6\beta2\gamma2$ $GABA_A$ Receptors

TBOB, lindane, TBPS, dieldrin, PTX, and PTZ inhibited [^{3}H] EBOB binding in a concentration dependent manner in all receptor types studied (TBOB was only examined in human $\alpha1\beta2\gamma2$ and $\alpha2\beta2\gamma2$ receptors). Competition curves for all 4 receptor types are shown in Figure 4. The rat cerebellum, human $\alpha1\beta2\gamma2$, and human $\alpha2\beta2\gamma2$ plots are representative of one of 3 or 4 experiments, each performed in triplicate. The rat $\alpha6\beta2\gamma2$ plot is representative of 1 of 2 experiments, each also performed in triplicate. Table 2 summarizes [^{3}H] EBOB competition data in all 4 receptor types. Each K_i and Hill slope (n_H) value is the average of 3 or 4 experiments ± S.E.M. in rat cerebellum, human $\alpha1\beta2\gamma2$, and human $\alpha2\beta2\gamma2$ receptors. In rat $\alpha6\beta2\gamma2$ receptors, the affinities and Hill slopes represent the average of 2 experiments ± S.E.M.

Table 2. Summary of Competition of [^{3}H] EBOB Binding by Convulsant Drugs in Rat Cerebellum, Human $\alpha1\beta2\gamma2$, Human $\alpha2\beta2\gamma2$, and Rat $\alpha6\beta2\gamma2$ $GABA_A$ Receptors. K_i ± s.e.m. are given in nM followed by n_H ± s.e.m. in parenthesis.

	Rat Cerebellum	Human $\alpha1\beta2\gamma2$	Human $\alpha2\beta2\gamma2$	Rat $\alpha6\beta2\gamma2$
TBOB	Not studied	93 ± 17 (0.80 ± 0.04)	79 ± 24 (0.73 ± 0.08)	Not studied
Lindane	276 ± 54 (0.85 ± 0.10)	137 ± 63 (0.77 ± 0.06)	155 ± 39 (0.90 ± 0.05)	35 ± 5 (0.64 ± 0.16)
TBPS	755 ± 40 (0.92 ± 0.09)	274 ± 145 (0.66 ± 0.07)	269 ± 32 (0.81 ± 0.08)	139 ± 77 (0.78 ± 0.19)
Dieldrin	636 ± 97 (1.09 ± 0.07)	327 ± 7 (0.77 ± 0.08)	693 ± 219 (0.78 ± 0.03)	302 ± 182 (0.68 ± 0.03)
PTX	556 ± 104 (0.95 ± 0.13)	3030 ± 1370 (0.79 ± 0.05)	2110 ± 610 (0.96 ± 0.06)	2110 ± 1480 (0.86 ± 0.19)
PTZ	661,000 ± 70,300 (0.85 ± 0.03)	268,000 ± 66,700 (0.91 ± 0.09)	267,000 ± 11,000 (1.05 ± 0.03)	2,490,000 ± 982,000 (0.80 ± 0.16)

In rat cerebellum, the K_i values were roughly 0.3 μM for lindane, 2-3 fold higher for TBPS, dieldrin, and PTX, and close to 1 mM (2400 fold higher) for PTZ. The Hill slopes were

all near unity, indicating competition at one site with no cooperativity. The rank order of potency was: lindane ≥ dieldrin ≈ PTX ≈ TBPS » PTZ.

In human α1β2γ2 receptors, TBOB, lindane, TBPS, dieldrin, PTX, and PTZ resulted in concentration dependent inhibition of [^{3}H] EBOB binding. The rank order of potency was as follows: TBOB ≈ lindane ≥ dieldrin ≈ TBPS > PTX » PTZ. The affinities of TBOB and lindane were highest and in the 0.1 μM range, followed by TBPS and dieldrin, with affinities roughly 3 fold lower. Next followed PTX, with an affinity roughly 30 fold lower than the most potent compounds, and finally, PTZ, with an affinity in the 0.3 mM range (roughly 2000-3000 fold lower than lindane or TBOB).

In human α2β2γ2 receptors, the affinity of TBOB was highest and near 0.1 μM, followed by lindane, with an affinity 2 fold lower, TBPS (3 fold lower affinity), dieldrin (10 fold lower affinity), PTX (affinity 27 fold lower), and finally, PTZ, with an affinity in the 0.3 mM range (3400 fold lower than lindane). The rank order of potency was as follows: TBOB ≥ lindane ≥ TBPS ≥ dieldrin ≥ PTX » PTZ.

Most Hill coefficients were near unity in α1β2γ2 and α2β2γ2 receptors, with the exception of TBPS in α1β2γ2 receptors and TBOB in α2β2γ2 receptors, which had Hill slopes near 0.7. This might indicate an allosteric effect, an additional affinity state, or an additional binding site that was not detected from the [^{3}H] EBOB direct binding Scatchard analysis. The criteria used by our laboratory was that a Hill slope within roughly 20% of unity was assumed to be close enough to unity to assume a single binding site.

Competition studies were also performed with dieldrin, lindane, TBPS, PTX, and PTZ in rat α6β2γ2 receptors. Only two experiments were performed. Because of a low receptor expression, nonspecific binding was about 60 – 70 % of total binding and bound specific counts were very low (200 – 300 cpm range). With such low specific binding, any experimental error

could have made a substantial difference in the IC_{50} or Hill slope of the logistic curves that best fit the points. The primary focus of these data was to compare the rank order of potency to the other receptor subtypes and not absolute numbers. Thus, the K_i values of $\alpha6\beta2\gamma2$ receptors were not compared to those of the other receptor types. The affinities were ranked as follows: lindane ≥ TBPS ≥ dieldrin ≥ PTX » PTZ.

The rank order of potency of competing compounds in the different receptor subtypes is summarized:

cerebellum: lindane ≥ TBPS ≈ dieldrin ≈ PTX » PTZ

$\alpha1\beta2\gamma2$: TBOB ≈ lindane ≥ TBPS ≈ dieldrin > PTX » PTZ

$\alpha2\beta2\gamma2$: TBOB ≥ lindane ≥ TBPS ≥ dieldrin ≥ PTX » PTZ

$\alpha6\beta2\gamma2$: lindane ≥ TBPS ≥ dieldrin ≥ PTX » PTZ

Even though there were some subtle differences between the different receptor subtypes, the general rank order of potency was comparable, with TBOB and lindane being the most potent compounds, followed by TBPS, dieldrin, and PTX, and PTZ being least potent.

To see if any significant differences existed in the K_i values of each convulsant drug between cerebellum, $\alpha1\beta2\gamma2$, and $\alpha2\beta2\gamma2$ receptors, statistical analysis was performed. The one-way ANOVA followed by Tukey's comparison resulted in the following significant differences:

TBPS:	rat cerebellum and human $\alpha1\beta2\gamma2$	$p < 0.05$
	rat cerebellum and human $\alpha2\beta2\gamma2$	
PTZ:	rat cerebellum and human $\alpha1\beta2\gamma2$	$p < 0.01$
	rat cerebellum and human $\alpha2\beta2\gamma2$	

Subunit dependent differences of [^{3}H] EBOB competition were seen with TBPS and PTZ. Both TBPS and PTZ have significantly higher inhibitory affinities in $\alpha1/2\beta2\gamma2$ receptors compared to cerebellum.

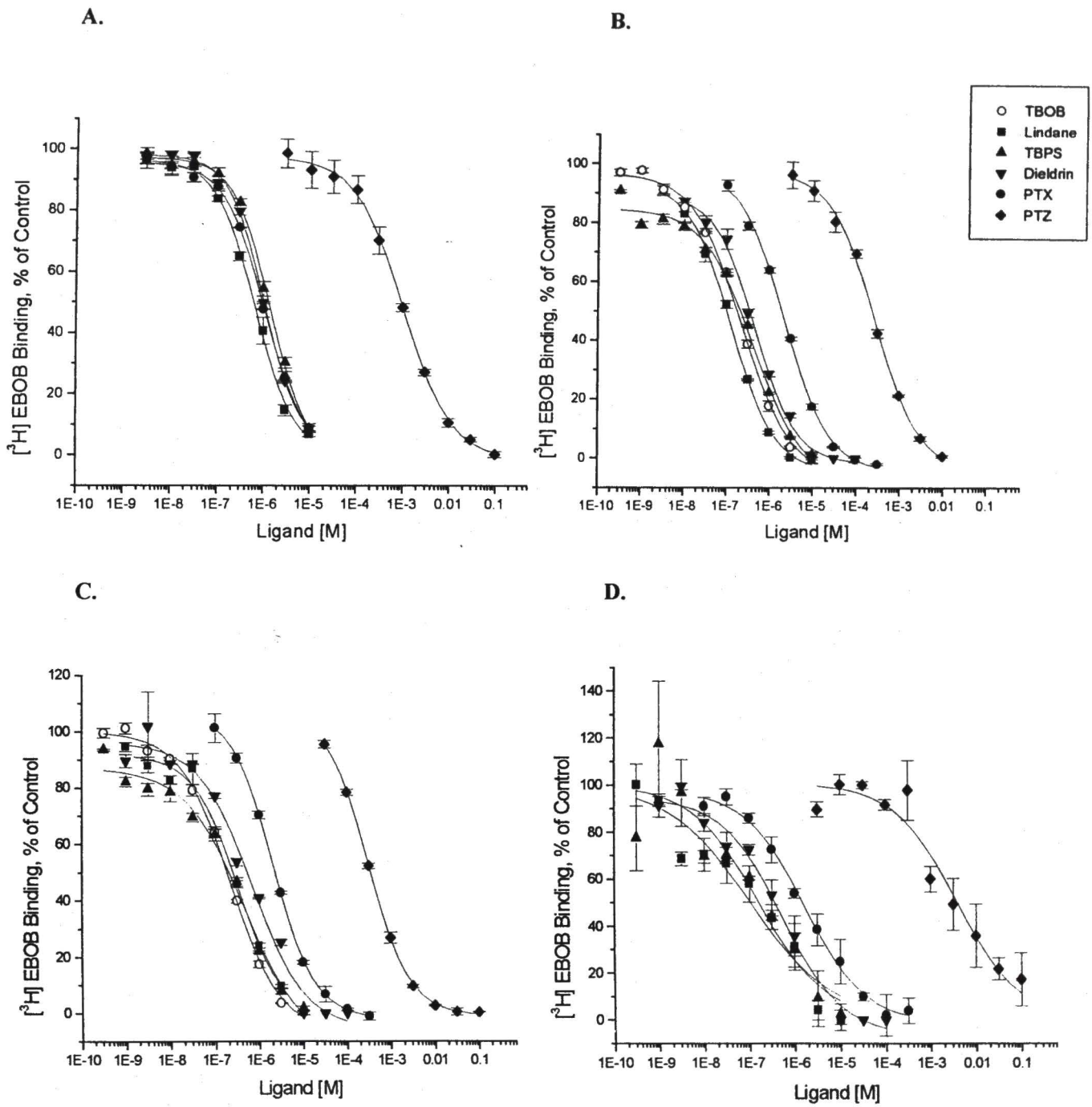


Figure 4. Competition of [^{3}H] EBOB Binding by Convulsant Compounds in (A) Rat Cerebellum, (B) Human $\alpha1\beta2\gamma2$ Receptors, (C) Human $\alpha2\beta2\gamma2$ Receptors, (D) Rat $\alpha6\beta2\gamma2$ Receptors. TBOB, lindane, TBPS, dieldrin, PTX, and PTZ inhibited [^{3}H] EBOB binding in a concentration-dependent manner. Inhibitory affinities varied from the low μM to the low mM range. Points of each curve were representative of one experiment performed in triplicate ± s.e.m. 2 experiments were performed in rat $\alpha6\beta2\gamma2$ and 3-4 experiments in the other receptor subtypes.

[^{35}S] TBPS Competition Binding in Human $\alpha1\beta2\gamma2$ $GABA_A$ Receptors

For comparison to [^{3}H] EBOB, we also investigated [^{35}S] TBPS binding in human $\alpha1\beta2\gamma2$ receptors. Figure 5, Panel A, shows that TBPS inhibited [^{3}H] EBOB in a concentration-dependent manner at one site in rat cerebellum. A comparable inhibition was obtained in the other receptor subtypes studied. Figure 5, Panel B, shows curves for the inhibition of [^{35}S] TBPS binding by TBPS, dieldrin, PTX, and PTZ. For comparison, [^{3}H] EBOB inhibition by the same compounds is illustrated in the same receptor subtype in Figure 5, Panel C. Table 3 summarizes the IC_{50} values and Hill slopes. TBOB and lindane were not examined.

Table 3. Competition of [^{35}S] TBPS by Convulsant Drugs in Human $\alpha1\beta2\gamma2$ Receptors.
(n = 2 experiments)

	Average of Individual Experiments One-Site Logistic Fits	
	IC_{50} ± s.e. (nM)	n_H ± s.e.
TBPS	164 ± 8	1.09 ± 0.06
Dieldrin	600 ± 279	0.72 ± 0.12
PTX	1300 ± 55	0.90 ± 0.06
PTZ	99,700 ± 29,400	1.09 ± 0.07

The main purpose of this study was to compare the rank order of potency from [^{35}S] TBPS inhibition to that of [^{3}H] EBOB inhibition in human $\alpha1\beta2\gamma2$ receptors. Even though subtle differences existed, a similar rank order of potency was seen:

[^{35}S] TBPS: TBPS ≥ dieldrin ≥ PTX » PTZ

[^{3}H] EBOB: TBPS ≈ dieldrin > PTX » PTZ

For [^{35}S] TBPS competition, TBPS had IC_{50} values in the 0.1-0.2 μM range, followed by dieldrin, with an affinity roughly 4-fold lower, then by PTX with an affinity around 10-fold lower than the most potent compound, and finally, PTZ, with an affinity 600-800 lower than TBPS. There was a slight difference in the affinity of some of the compounds when comparing both radioligands in human $\alpha1\beta2\gamma2$ receptors. PTX and PTZ appeared to be slightly more potent in

displacing [^{35}S] TBPS binding compared to [^{3}H] EBOB binding (at least 2-3 fold for both compounds). Most Hill slopes were near unity, indicating displacement of [^{35}S] TBPS binding at one site. The exception was dieldrin, which had a Hill slope near 0.7. This shallower slope could have been a result of the correction applied to the paradoxal increase in [^{35}S] TBPS binding at high dieldrin concentrations. These results suggest that [^{3}H] EBOB and [^{35}S] TBPS competition by convulsant compounds are very comparable, and that both ligands likely bind to the same region or to regions with overlapping domains.

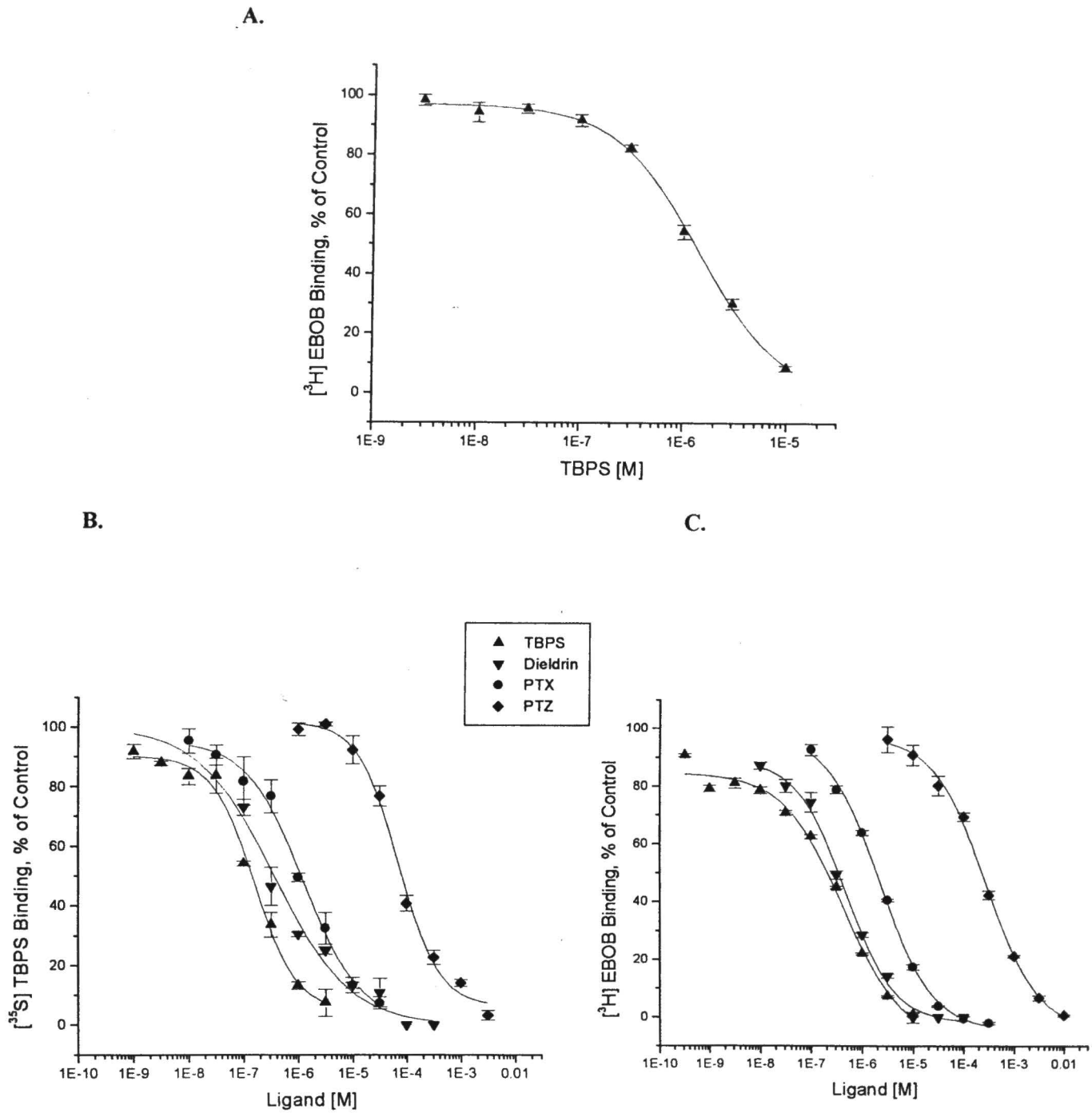


Figure 5. (A) Concentration-Dependent Inhibition of [^{3}H] EBOB by TBPS in Rat Cerebellum. (B) Concentration-Dependent Competition of [^{35}S] TBPS Binding and (C) [^{3}H] EBOB Binding by TBPS, Dieldrin, PTX, and PTZ in Human $\alpha1\beta2\gamma2$ $GABA_A$ Receptors. Inhibitory affinities and rank order of potency were comparable and ranged from low μM to low mM. For [^{3}H] EBOB, points were representative of one of 3-4 experiments ± s.e.m. For [^{35}S] TBPS, points were representative of 2 experiments ± s.e.m.

The Effect of GABA on [^{3}H] EBOB Binding in Human $\alpha1\beta2\gamma2$ Receptors

Because GABA has previously been shown to have stimulatory and inhibitory effects on [^{35}S] TBPS binding (Luddens and Korpi, 1995; Pregenzer et al., 1993; Im et al., 1994), we wanted to investigate its effect on [^{3}H] EBOB binding. GABA had a biphasic effect on subsaturating [^{3}H] EBOB concentrations (3 nM) in human $\alpha1\beta2\gamma2$ receptors. Figure 6 shows that low concentrations of GABA stimulated [^{3}H] EBOB binding, while high concentrations inhibited binding. At a GABA concentration of 31.6 nM, [^{3}H] EBOB binding was at 100% of control level. Stimulation up to about 250% of control occurred at a GABA concentration of 10 μM, followed by inhibition at GABA concentrations greater than 10 μM. The highest concentration tested, 100 mM, inhibited [^{3}H] EBOB binding to roughly 35% of control. Higher concentrations of GABA were not tested because of physiological irrelevance, so it was not known if complete inhibition of [^{3}H] EBOB binding would have occurred. The EC_{50} value of stimulation was 1.01 ± 0.12 μM (from roughly 100% to 250% of control, n=4) and the IC_{50} value of inhibition was 120 ± 5 μM (from roughly 250% to 35% of control, n=3). There was a 120-fold difference between stimulatory and inhibitory affinities.

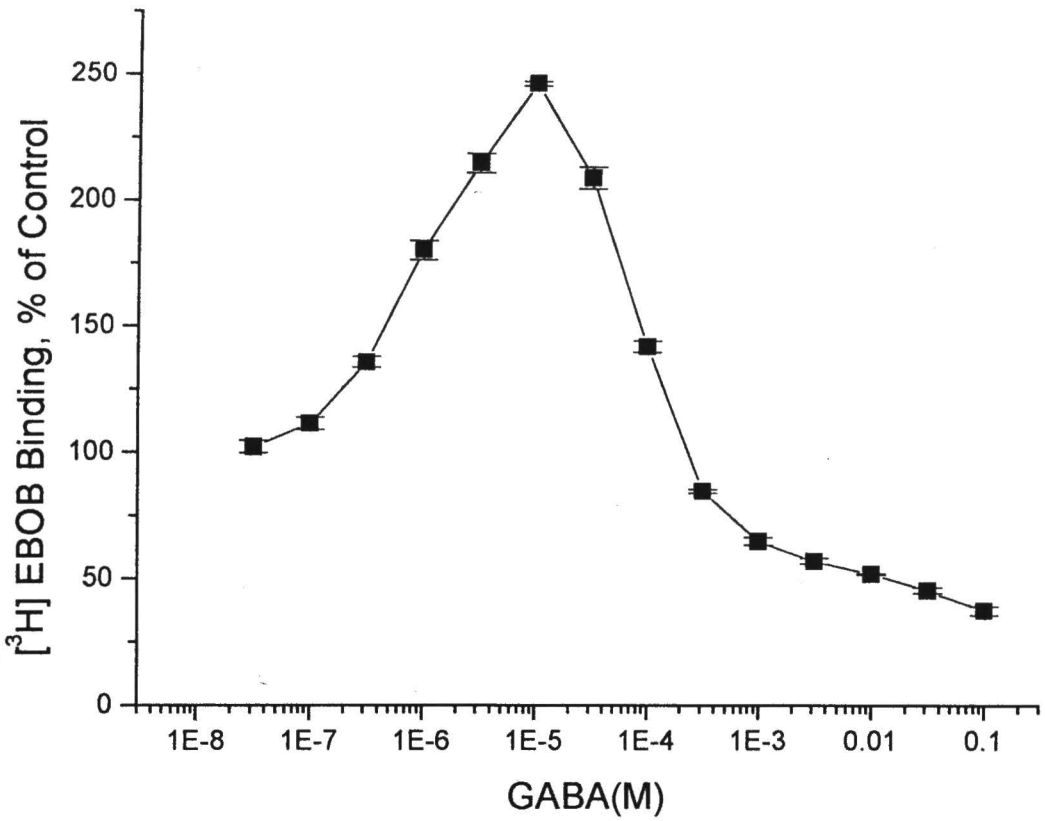


Figure 6. Modulation of [^{3}H] EBOB Binding by GABA in Human $\alpha1\beta2\gamma2$ $GABA_A$ Receptors. At low concentrations, GABA stimulated [^{3}H] EBOB binding to about 250% of control. Concentrations greater than 10 μM GABA inhibited [^{3}H] EBOB binding. The difference between inhibitory and stimulatory affinities was 120-fold. The [^{3}H] EBOB concentration was at a subsaturating level (3 nM). Points ± s.e.m represented one of three experiments.

TBOB Inhibition of GABA-Induced Chloride Current in Human $\alpha 1\beta 2\gamma 2$ $GABA_A$ Receptors

From our binding studies, we suspect that EBOB likely binds to the noncompetitive convulsant site. Next, we wanted to determine its functional effect on $GABA_A$ receptors. Because EBOB is not available commercially, it was replaced by TBOB, the closest available compound in structural similarity, to examine the functional effect on GABA-mediated chloride current. Figure 7, Panel A, is a representative current trace of the inhibition of 10 μM GABA mediated chloride current by 1μM TBOB applied for 20 seconds a total of 12 times consecutively (not corrected for time dependent changes in current amplitude). The average time dependent change in current amplitude was determined to be 82.6 ± 8.0 % of control (n = 6). At the 12^{th} application, 1 μM TBOB inhibited the steady state current amplitude to about 33% of control. In some cells, the initial 20 second TBOB application caused a stimulation above control in peak current. This stimulation was seen in 6 different cells at TBOB concentrations of 0.01, 0.1, 0.3, and 1 μM, and ranged from 103% to 140% of the control current amplitude. In general, an increase in current decay was also observed in some cells during the initial 20 second TBOB application over all TBOB concentrations except 0.01 μM.

We found that TBOB functionally inhibited GABA mediated current. The curve representing the inhibition of 10 μM GABA-mediated chloride current by varying TBOB concentrations is represented in Figure 7, Panel B. The current amplitude as a percentage of control represented the steady state current after 12 consecutive 20 second TBOB applications corrected for time dependent changes (n=3-6 cells at each TBOB concentration). The TBOB concentration of 0.01 μM stimulated the steady state current amplitude to 111% of control when corrected for time dependent changes. The IC_{50} value for TBOB inhibition of 10 μM GABA induced current was 0.216 μM and the Hill slope was 0.74.

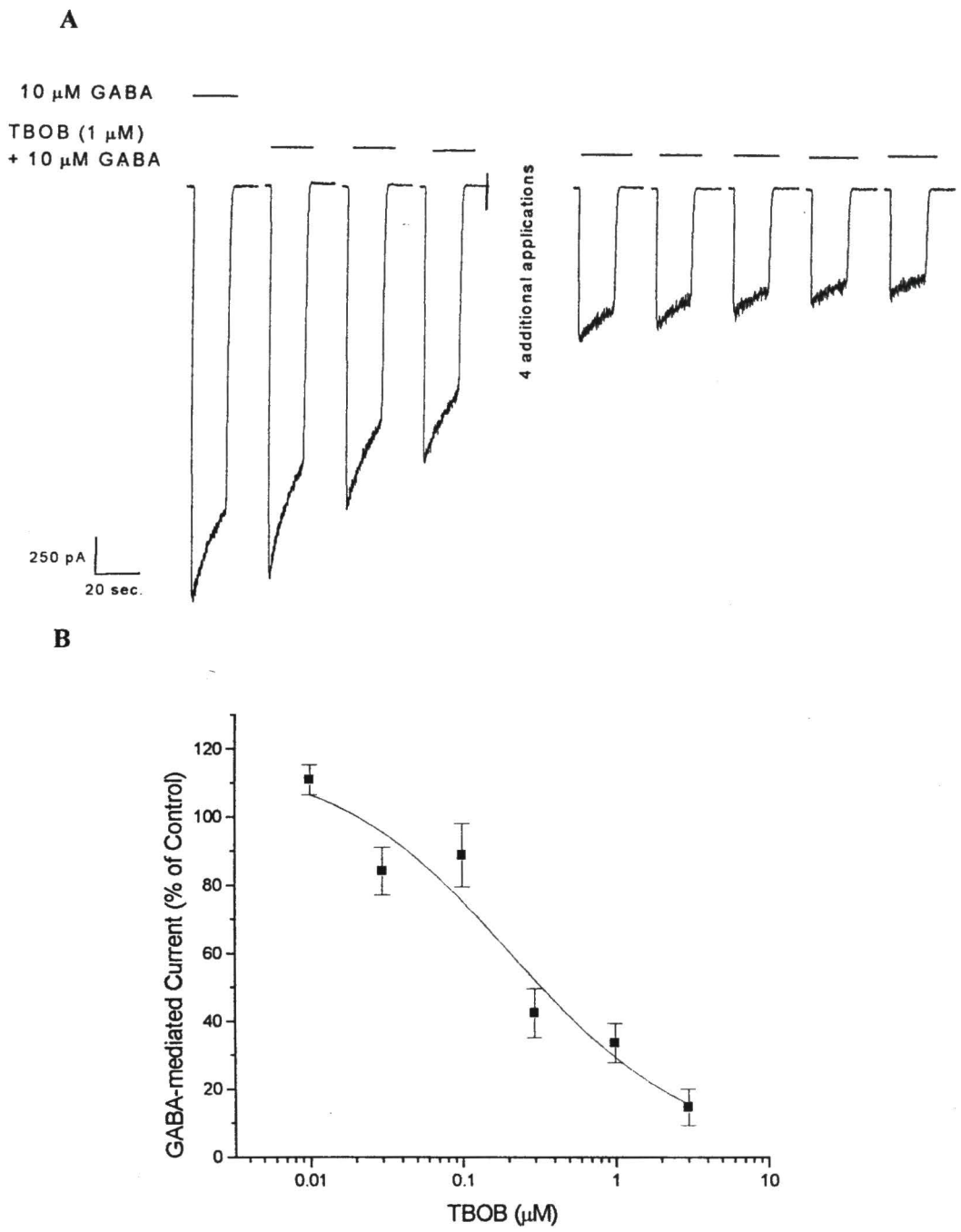


Figure 7. (A) Current Traces of 1 μM TBOB Inhibition of 10 μM GABA-Induced Chloride Current in Human α1β2γ2 Receptors. 1 μM TBOB was applied consecutively for 20 seconds a total of 12 times and inhibited the steady state current amplitude to 33% of control with the 12th application. **(B) TBOB Inhibition Curve of 10 μM GABA-Mediated Current in Human α1β2γ2 $GABA_A$ Receptors.** IC_{50} = 0.216 μM. At 0.01 μM, TBOB slightly stimulated the steady state current amplitude. Error bars represent s.e.m. and n = 3-6 cells at each TBOB concentration.

CHAPTER IV. DISCUSSION

Our research has shown that [^{3}H] EBOB is a useful radioligand to study the noncompetitive convulsant site in recombinant receptors and that it binds to the TBPS site or at least to a region sharing domains with the TBPS site. From our saturation and competition data, we have shown that [^{3}H] EBOB binding affinity and competition of [^{3}H] EBOB binding is dependent on subunit type. Generally, our saturation, competition, and modulation studies yielded comparable results to literature available on [^{3}H] EBOB, [^{3}H] TBOB, and [^{35}S] TBPS binding. TBOB's functional inhibition of GABA-mediated current correlated to binding data.

Comparison of [^{3}H] EBOB Binding to [^{3}H] TBOB and [^{35}S] TBPS Binding in Native Tissue and Recombinant Receptors: Saturation Studies

Our studies with [^{3}H] EBOB resulted in affinities that were higher than [^{35}S] TBPS and [^{3}H] TBOB affinities, and comparable to, but generally slightly lower than affinities of [^{3}H] EBOB from the literature. [^{3}H] TBOB and [^{35}S] TBPS affinities from the literature (Lawrence et al., 1985; van Rijn et al., 1990; Hawkinson and Casida, 1992; Sakurai et al., 1994; Pericic et al., 1998; Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Holland et al., 1989; Pomes et al., 1993; Korpi and Luddens, 1993; Im et al., 1994; Dillon et al., 1995; Slaney, et al., 1995) were generally lower than [^{3}H] EBOB affinities from the literature (Hawkinson and Casida, 1992; Cole and Casida, 1992; Huang and Casida, 1996; Kume and Albin, 1994) and from our studies, usually between 5-100 fold depending on native tissue type or receptor subtype. In our studies, [^{3}H] EBOB bound to saturable, single high affinity sites in rat cerebellum, rat $\alpha6\beta2\gamma2$ receptors, human $\alpha1\beta2\gamma2$, and human $\alpha2\beta2\gamma2$ receptors, with affinities ranging from 3-9 nM (Table1, Figure 3). Our criteria for establishing the single binding site conclusion was based on the high correlation coefficients from the linear fits of the Scatchard plots. The highest affinities were in

the cerebellum and α6β2γ2 receptors (3-4 nM), and the lowest, in α1β2γ2 and α2β2γ2 receptors (9 nM). Similar K_d values were obtained for rat cerebellum and α6β2γ2 receptors possibly because of the unique expression of the α6 subunit in cerebellar granule cells, which are at the same time, the most numerous and smallest neurons in the CNS (Wisden et al., 1996).

This difference in affinity between the recombinant receptors is likely due to the α subunit type (α6 vs. α1/2), and not species differences. It should be noted that attempts to examine [^{3}H] EBOB binding in rat α1β2γ2 receptors were made, but with no success because of very low receptor expression. We would have expected the data to show nearly identical affinities between rat and human α1β2γ2 receptors, indicating that the difference in affinities between the α6 vs. α1/2-containing receptors would have been due to the α subunit type and not species differences. This is supported by the finding that the amino acid sequence identity between rat α1 and bovine α1 was 99% (Khrestchatisky et al., 1989), and the identity between human α1 and bovine α1 was also 99% (Schofield et al., 1989). From this, we can conclude that the rat and human α1 amino acid sequences must be highly homologous with an identity near 99%.

In addition, we also wanted to examine [^{3}H] EBOB binding in receptors lacking the α subunit, but did not succeed because we could not achieve any specific binding in β2γ2 receptors. This was most likely due to a very low receptor expression. Thus, we were not able to examine the effect of the lack of the α subunit on [^{3}H] EBOB binding affinity. Experiments performed in our laboratory have shown dieldrin and PTX to have higher functional affinities in receptors lacking the α subunit (unpublished observations; Bell-Horner, in press; Bell-Horner, 1999). [^{3}H] EBOB might also have a higher binding affinity in β2γ2 receptors relative to the receptors containing α subunits. If these experiments could have been performed, we could have

determined if this higher affinity was due to binding or to another aspect affecting the functional gating of the channel.

To the best of our knowledge, [^{3}H] EBOB has only been studied in vertebrate tissue and insect head and not in recombinant receptors. In membrane preparations from brains of bovine, human, dog, mouse, chicken, and fish, and fly heads, [^{3}H] EBOB bound with K_d values ranging from 0.4-4.4 nM (Hawkinson and Casida, 1992; Cole and Casida, 1992). In membrane preparations from cultured cerebellar granule neurons and rat cerebellum, [^{3}H] EBOB bound with a comparable affinity of 0.5-0.6 nM (Huang and Casida, 1996), affinities 6-8 fold higher than those obtained by us in rat cerebellum and $\alpha6\beta2\gamma2$ receptors. Just as Huang and Casida (1996) found no significant difference in [^{3}H] EBOB binding between rat cerebellum and cells of the granule layer, neither did we between rat cerebellum and $\alpha6\beta2\gamma2$ receptors (representative of the granule cell layer due to the significant expression in this layer). It is also interesting to note that in rat cerebellum, Huang and Casida (1996) determined the total number of [^{3}H] EBOB binding sites to be 5-fold lower than what we determined. This discrepancy in K_d and B_{max} could have been due to different assay conditions, different age and species of rats, different tissue preparations that may have washed out different amounts of GABA, and/or the use of bicuculline in our assay but not in Huang and Casida's. By blocking GABA binding sites in rat cerebellum in our experiments, bicuculline roughly doubled [^{3}H] EBOB specific bound radioactivity, thus indicating an increase in [^{3}H] EBOB binding sites. This result might contradict our results of GABA modulation of [^{3}H] EBOB binding, where GABA concentrations lower than 100 μM stimulated [^{3}H] EBOB binding. Based on this result, bicuculline would be expected to decrease [^{3}H] EBOB binding. The discrepancy seen here may be due to differences in $GABA_A$ receptor subtype. The $\alpha6\beta2\gamma2$ receptor subtype of the cerebellum might contribute to this property, which appears to be opposite to that seen in $\alpha1\beta2\gamma2$ receptors. Because the cerebellum likely contains a

substantial amount of α6β2γ2 receptors, [^{3}H] EBOB binding sites might be inhibited by GABA concentrations less than 100 μM, which are stimulated in α1β2γ2 receptors at these same concentrations. Other studies have shown regional brain modulation of [^{35}S] TBPS binding sites by GABA and bicuculline (Korpi and Luddens, 1993; Vale et al., 1997; Korpi et al., 1996). [^{3}H] EBOB binding was also examined with autoradiography in rat brain (Kume and Albin, 1994). In cortical lamina IV, it bound with a K_d of 4.6 nM, comparable to our affinity in cerebellar membrane preparations. In all of the previously mentioned studies, as in ours, Scatchard analysis revealed [^{3}H] EBOB binding to a single, saturable site.

[^{3}H] EBOB binds with a higher affinity than [^{3}H] TBOB to mammalian $GABA_A$ receptors. Autoradiographic and membrane preparation studies of [^{3}H] TBOB binding to rat and bovine brain occurred at single, saturable sites with K_d values of 6-61 nM (Lawrence et al., 1985; van Rijn et al., 1990; Hawkinson and Casida, 1992; Sakurai et al., 1994), affinities comparable to ours with [^{3}H] EBOB, but generally 10-fold lower. Only one study has examined [^{3}H] TBOB in recombinant receptors. Pericic et al. (1998) found [^{3}H] TBOB to bind to a single saturable site with a K_d of 47 nM in rat α1β2γ2 receptors, an affinity roughly 5 fold lower than that obtained by us for [^{3}H] EBOB in human α1β2γ2 receptors. These results show that [^{3}H] EBOB binds with a higher affinity than [^{3}H] TBOB to mammalian $GABA_A$ receptors.

Our data revealed that [^{3}H] EBOB binds to $GABA_A$ receptors with a higher affinity than [^{35}S] TBPS. The majority of saturation studies suggest that [^{35}S] TBPS binds to brain membrane preparations at a single, saturable site with K_d values ranging from 15-55 nM (Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Holland et al., 1989; Hawkinson and Casida, 1992; Pomes et al., 1993). In rat cerebellar membranes, [^{35}S] TBPS had an affinity of 21 nM (Slaney, et al., 1995; Zezula, et al., 1996), roughly 5-fold lower than that obtained with [^{3}H] EBOB in our studies. In intact cultured cerebellar granule cells, [^{35}S] TBPS had an affinity of 42-100 nM

(Gallo et al., 1985; Pomes et al., 1993). Gallo et al. (1985) argued that [^{35}S] TBPS had a lower affinity in intact cells, while Pomes et al. (1993) argued that affinities were the same in membrane preparations or intact cells of the granule cell layer. A lower affinity in intact cells might be more representative of a functional state because the cells had not been disrupted (Gallo et al., 1985). Ito and Ho (1994) examined [^{35}S] TBPS binding in rat cortical synaptoneurosomes, which contain both pre- and post-synaptic vesicular structures, and found a K_d of 76 nM, a debatable result if concluding that [^{35}S] TBPS affinity is lower in synaptoneurosomes than membrane preparations. One study has shown [^{35}S] TBPS to bind to two distinct sites with different affinities in rat and chicken cerebrum (Tehrani et al., 1985). In both tissue types, high affinity binding was near 1 nM while low affinity binding was roughly 100-200 fold lower. Receptor expression ranged from roughly 100 fmol/mg protein in the high affinity states of both species to about 17,000 fmol/mg protein in the low affinity states. These low affinity binding sites were roughly 5-12 fold more numerous than those obtained from some of the previously mentioned studies and our [^{3}H] EBOB study in rat cerebellum.

Our results revealed that [^{3}H] EBOB binding affinity is dependent on receptor subtype, namely on α subunit isoform (α1/2 vs. α6). [^{35}S] TBPS binding has also been found to be subunit specific. Unlike [^{3}H] EBOB and [^{3}H] TBOB, [^{35}S] TBPS binding and its modulation by a variety of compounds have been studied in recombinant receptors. [^{35}S] TBPS bound to a single site with K_d values ranging from 8-135 nM in α1/3/4/5/6β2/3γ2/3, α1/3/6β2/3, β3γ2, and β3 receptors (Korpi and Luddens, 1993; Im et al., 1994; Dillon et al., 1995; Luddens et al., 1994; Slaney et al., 1995; Zezula et al., 1996; Ebert et al., 1996). Affinities of [^{35}S] TBPS binding were roughly equal in α1β2γ2 and α6β2γ2 receptors (41-49 nM) (Korpi and Luddens, 1993; Im et al., 1994; Dillon et al., 1995), unlike our [^{3}H] EBOB affinities, which significantly differed by 3-fold. The lack of the γ2 subunit in α6, α1,α3 and β3 (no α) containing receptors did not have a

substantial effect on affinity (Im et al., 1994; Slaney et al., 1995; Zezula et al., 1996). The substitution of γ3 for γ2 in α5 containing receptors increased the affinity 2-fold, but not significantly enough to conclude that the TBPS site is affected to any significant level by the γ subunit (Luddens et al., 1994). A higher TBPS affinity was seen in α1β3γ2 compared to α1β2γ2 receptors (Korpi and Luddens, 1993; Zezula et al., 1996). In the β2 containing receptors, the α3 containing receptors possessed the lowest affinities, while in the β3 containing receptors, α1 containing receptors possessed higher affinities than α4 or α5 receptors (Im et al., 1994; Luddens et al., 1994; Zezula et al., 1996; Ebert et al., 1996). From these results, it can be inferred that [^{35}S] TBPS binding is dependent on α and β subunit subtype and not significantly affected by the type of or lack of the γ subunit.

Our data revealed that [^{3}H] EBOB binding affinity in receptors containing the same β and γ subunits differed between α1/2 and α6 isoforms. We also found differences between [^{3}H] EBOB affinity in the cerebellum, which is composed of various subunits, and α1/2β2γ2 receptors, but not α6β2γ2 receptors. This indicates that properties of the [^{3}H] EBOB binding site in cerebellar receptors are comparable to those of α6β2γ2 receptors. Another possibility is that the cerebellum contains a large proportion of α6β2γ2 receptors relative to other receptor subtypes. It should also be noted that bicuculline was used in the cerebellar assays, but not in any of the assays with recombinant receptors. Because bicuculline is a competitive antagonist at the GABA binding site, it allosterically modulates the noncompetitive convulsant site. This allosteric effect of bicuculline in native receptors may have also been responsible for the differences observed between [^{3}H] EBOB affinities in cerebellum and α1/2β2γ2 receptors. Zezula et al. (1996) found that receptors expressing αβγ and αβ subunits assembled more efficiently (higher B_{max}) with higher affinities for [^{35}S] TBPS compared to receptors expressing βγ or just β subunits.

This line of reasoning applies to our attempt to perform binding studies in β2γ2 receptors with no success. Failure to achieve any specific binding was most likely due to very low receptor expression. HEK cells expressing α3β1/2γ2/3 and α5β1/2γ2/3 receptors did not bind [^{35}S] TBPS well (low B_{max}), while cells expressing α1β1/2/3γ2/3, α2β1/2/3γ2/3, α3β3γ2/3, α5β3γ2/3, and α6β1/2/3γ2 receptors did bind [^{35}S] TBPS to levels sufficient enough for binding studies (Luddens et al., 1994; Luddens and Korpi, 1995). Note that all receptors containing α1 and α2 subunits recognized [^{35}S] TBPS binding, but that only the receptors with an α3 or α5 that also expressed a β3 subunit and not a β1/2 had sufficient [^{35}S] TBPS binding. Im et al. (1994) were able to achieve a very high level of [^{35}S] TBPS binding in α3β2γ2 receptors in SF-9 cells, so failure to get significant levels of binding by Luddens and Korpi (1995) was likely due to their expression system and not to the specific combination of subunits. The study by Luddens and Korpi signifies the subunit dependent difference of [^{35}S] TBPS binding based on α and β subunit subtypes and their different combinations during assembly. In general, specific subunit dependent binding of [^{35}S] TBPS has not been as extensively studied as subunit differences of [^{35}S] TBPS modulation by allosteric compounds.

Comparison of [^{3}H] EBOB to [^{3}H] TBOB and [^{35}S] TBPS Competition by Convulsant Compounds

In our study, dieldrin, lindane, TBOB, TBPS, PTX, and PTZ inhibited [^{3}H] EBOB binding in rat cerebellum, human α1β2γ2, human α2β2γ2, and rat α6β2γ2 receptors in a concentration dependent manner (Figure 4). Significant differences existed in inhibitory affinities of TBPS and PTZ between cerebellum and α1/2β2γ2 receptors. Both TBPS and PTZ had significantly higher inhibitory affinities in α1/2β2γ2 receptors compared to cerebellar receptors. This could have been due to the lower binding affinity of [^{3}H] EBOB in α1/2β2γ2 receptors compared to cerebellum, thus requiring less competing compound to inhibit EBOB binding to a

certain percentage of control. Even though receptors containing the subunit combinations that we examined have comparable pharmacological properties at the noncompetitive convulsant binding site, differences do exist in the inhibition of [^{3}H] EBOB binding by some competitive drugs.

In our experiments, the rank order of potency of the convulsant drugs in displacing [^{3}H] EBOB binding within each receptor subtype was generally similar (Table 2). In general, TBOB had the highest affinity followed by lindane, TBPS, dieldrin, PTX, and PTZ. TBPS, dieldrin, and PTX were comparable in affinities, with PTX having a lower affinity in the recombinant receptors. Comparing inhibitory affinities in cerebellum to $\alpha 1/2\beta 2\gamma 2$ receptors, PTX was more potent in rat cerebellum, but not to a significant level. TBPS and PTZ were least potent in rat cerebellum. Dieldrin and lindane were equally potent among cerebellum and $\alpha 1/2\beta 2\gamma 2$ receptors. In general, Hill coefficients were near, but just under, unity. This likely indicates competition at one site. The exceptions were TBPS in $\alpha 1\beta 2\gamma 2$ receptors and TBOB in $\alpha 2\beta 2\gamma 2$ receptors, which had Hill slopes near 0.7. These shallow Hill slopes may indicate an allosteric effect, negative cooperativity, or inhibition of [^{3}H] EBOB at more than one site or more than one affinity state. The multiple site explanation may not be likely because we found [^{3}H] EBOB to bind to a single site in all receptor subtypes examined. This could be a valid explanation if competition binding revealed an additional site that saturation binding did not. This additional site could have the same affinity or a similar affinity to the site detected from saturation binding. Another possibility for the shallow Hill slopes is that the binding reactions may not have reached equilibrium in the competition studies, even though 1.5 hours of incubation time was allotted.

Our studies showed that TBPS, dieldrin, PTX, and PTZ displaced [^{35}S] TBPS binding in human $\alpha 1\beta 2\gamma 2$ receptors in a concentration-dependent manner with affinities and a rank order of potency similar to those seen with [^{3}H] EBOB (Figure 5, Table 3). The only small difference was that PTX and PTZ may have been slightly more potent in displacing [^{35}S] TBPS binding

compared to [^{3}H] EBOB binding. Hill slopes were near unity, indicating competition at one site, with the exception of dieldrin, which had a shallower Hill coefficient near 0.7. As mentioned in the results section, this may have been due to the correction used for the paradoxal increase in [^{35}S] TBPS binding at higher dieldrin concentrations. Therefore, the convulsant compounds studied displaced [^{35}S] TBPS and [^{3}H] EBOB binding with similar affinities and a rank order of potency in $\alpha1\beta2\gamma2$ receptors. The slightly higher affinities seen in [^{35}S] TBPS displacement may have been due to [^{35}S] TBPS being less potent than [^{3}H] EBOB. Based on these comparable results, we believe that [^{3}H] EBOB binds to the same site as [^{35}S] TBPS or at least to a site within the same region that has overlapping domains.

Our [^{3}H] EBOB competition data has shown subunit differences for TBPS and PTZ. In general, our inhibitory affinities in cerebellum compared to those from the [^{3}H] EBOB literature. Our inhibitory affinities in recombinant receptors differed somewhat from those in native tissue for some convulsants as observed from our studies and from comparison to the literature. Competition of [^{3}H] EBOB binding by lindane, TBPS, and PTX in various vertebrate brain membranes and in an autoradiography study (PTX only) gave IC_{50} values comparable to ours in rat cerebellum, but up to 2-fold higher for lindane and 5-fold lower for TBPS (Cole and Casida, 1992; Kume and Albin, 1994). Insects were found to be more sensitive to lindane and PTX (lower IC_{50}) than vertebrates, and vertebrates, more sensitive to TBPS (Cole and Casida, 1992). [^{3}H] EBOB competition by convulsants in cultured rat granule neurons and rat cerebellum showed no differences in potencies between tissue types (Huang and Casida, 1996). Inhibitory affinities were very comparable to those obtained by us in rat cerebellum. Huang and Casida (1997) emphasized that competition of [^{3}H] EBOB by convulsant drugs showed no cellular or regional specificity, even in other brain regions examined, but that differences in GABA

modulation were seen. We did observe subunit specificity for competition of [^{3}H] EBOB binding by the convulsants TBPS and PTZ.

Modulation of [^{3}H] TBOB binding has only been investigated in a few studies (van Rijn et al., 1990; Pericic et al., 1998). [^{3}H] TBOB inhibition by convulsants in rat brain membranes resulted in inhibitory affinities that were comparable to or slightly different from ours (van Rijn et al., 1990). In rat $\alpha1\beta2\gamma2$ receptors, [^{3}H] TBOB was inhibited by TBOB and PTX with affinities comparable to ours for TBOB in human $\alpha1\beta2\gamma2$ receptors, but at least 10-fold higher (lower IC_{50}) for PTX (Pericic et al., 1998). Hill coefficients were near unity (Pericic et al., 1998).

Currently, [^{35}S] TBPS is the standard radioligand used to investigate other convulsant agents or allosteric modulators. [^{35}S] TBPS inhibition by EBOB, TBOB, TBPS, dieldrin, PTX, and PTZ was examined in native tissue (Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Holland et al., 1989; Hawkinson and Casida, 1992; Gallo et al., 1985; Pomes et al., 1993). Generally, our inhibitory affinities obtained from displacing [^{3}H] EBOB binding were lower (higher K_i) by 3-40 fold with the exception of PTZ, which was very comparable. The reason for these differences is likely due to the lower affinity of TBPS compared to EBOB, requiring less competitive drug to displace it, thus obtaining lower IC_{50} values (higher inhibitory affinities) for the convulsant drugs. Tehrani et al. (1985) found TBPS and PTX to inhibit [^{35}S] TBPS binding in rat and chicken cerebrum at two sites, with comparable IC_{50} values between tissue types. In general, the convulsant drugs discussed inhibited [^{35}S] TBPS binding in different tissues with comparable affinities between the tissues. Inhibitory affinities from [^{35}S] TBPS literature were generally similar but may have differed in absolute values to those obtained by our [^{3}H] EBOB and [^{35}S] TBPS studies, even though rank order of potency was generally the same. Particularly, in our $\alpha1\beta2\gamma2$ receptors, [^{35}S] TBPS inhibitory affinities were slightly lower (higher IC_{50}) for dieldrin, PTX, and TBPS, and higher (lower IC_{50}) for PTZ compared to the native tissue results

found in the literature. These differences could have been due to different tissues/receptor subtypes or assay conditions, and may suggest subunit dependent differences of [^{35}S] TBPS binding.

GABA Modulation of [^{3}H] EBOB, [^{35}S] TBPS, and [^{3}H] TBOB Binding

In addition to competition of [^{3}H] EBOB binding by convulsant drugs, we also wanted to examine EBOB's modulation by GABA at a subsaturating concentration and compare our results to those found for [^{35}S] TBPS. GABA modulation of radiolabled convulsant drug binding has been reported as both monophasic and biphasic. Various studies found GABA to inhibit [^{35}S] TBPS and [^{3}H] TBOB binding in a concentration-dependent fashion with no stimulation (Lawrence et al., 1985; van Rijn et al., 1990; van Rijn et al., 1995; Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Slaney et al., 1995; Ito and Ho, 1994). The modulation of [^{3}H] EBOB by GABA has only been examined in native tissue and has been found to be monophasic with only an inhibitory phase and no stimulation (Cole and Casida, 1992; Huang and Casida, 1996 & 1997). Pericic et al. (1998) found GABA to inhibit [^{3}H] TBOB binding in $\alpha1\beta2\gamma2$ receptors with no stimulation and an IC_{50} value of 0.4 μM. This result by [^{3}H] TBOB differed from ours with [^{3}H] EBOB in the same receptor subtype, where we saw both stimulation and inhibition (Figure 6). We found one study that showed GABA to modulate [^{35}S] TBPS binding in native tissue either biphasically or monophasically depending on brain region (Liljequist and Tabakoff, 1993).

GABA has also been found to modulate [^{35}S] TBPS binding biphasically, especially in recombinant receptors (Luddens and Korpi, 1995). [^{35}S] TBPS binding and its regulation by GABA were examined in various configurations consisting of $\alpha1/2/3/5/6$ and $\beta\gamma$ subunits (Luddens and Korpi, 1995). GABA modulation of [^{35}S] TBPS binding varied based on receptor subtype, with stimulation occurring in most receptor subtypes at concentrations varying from 100

nM to 1-10 μM, and inhibition occurring at higher GABA concentrations. Receptors expressing α1β2γ2 subunits showed [^{35}S] TBPS stimulation up to 160% at 1 μM GABA, and inhibition at greater concentrations (Korpi and Luddens, 1993; Luddens and Korpi, 1995). Maximal GABA stimulation of [^{3}H] EBOB binding in our α1β2γ2 receptors was 250% of control, higher than seen with [^{35}S] TBPS, and occurred at 10 μM, a GABA concentration 10-fold higher than with seen [^{35}S] TBPS modulation from Luddens and Korpi (1995). GABA stimulated [^{3}H] EBOB binding to a greater extent than [^{35}S] TBPS binding in α1β2γ2 receptors. Higher GABA concentrations were also required to inhibit [^{3}H] EBOB binding compared to [^{35}S] TBPS binding in α1β2γ2 receptors probably due to EBOB's higher affinity or a different allosteric interaction between the GABA binding sites and the EBOB site, which may not be identical to the TBPS site. The biphasic effect of GABA on [^{3}H] EBOB (at a subsaturating concentration of 3 nM) binding in α1β2γ2 receptors may be due to reasons involving binding affinity or the number of exposed sites. Low concentrations of GABA up to about 100 μM may reveal additional [^{3}H] EBOB binding sites by opening the chloride channel and allowing easier access for EBOB to bind to its site within the channel pore. Low GABA concentrations may also allosterically modulate the EBOB site so that its affinity for EBOB is increased. Higher GABA concentrations (greater than 100 μM) may cause desensitization, thus causing the channel pore to close and block access of EBOB to its binding site. Whether or not GABA concentrations greater than 100 mM result in complete EBOB binding inhibition remains to be determined. GABA modulation of [^{3}H] EBOB binding in other receptor subtypes should also be examined to see if subunit differences exist, as with [^{35}S] TBPS modulation. Experiments in cerebellum and α6β2γ2 receptors might help to explain the increase in [^{3}H] EBOB binding seen when GABA sites were blocked with bicuculline.

The effect of the γ2 subunit on GABA modulation of [^{35}S] TBPS binding has also been examined (Pregenzer et al., 1993; Im et al., 1994). GABA had a biphasic effect on [^{35}S] TBPS binding in α1β2, α3β2, and α6β2 receptors, causing enhancement and inhibition at nM and μM concentrations, respectively. The maximal enhancement of binding was a function of the α subunit type, with α3β2>α1β2>α6β2 (Im et al., 1994). GABA stimulated [^{35}S] TBPS binding in α1β2γ2, α3β2γ2, and α6β2γ2 receptors to a much lower extent, just slightly above 100% of control and up to 150% in α1β2γ2 receptors. In α1β2 receptors, stimulatory and inhibitory affinities seemed to differ just over 10-fold, while in α1β2γ2 receptors, maximal stimulation occurred at about 1 μM, and the inhibitory affinity was 10-fold lower than in α1β2 receptors (Im et al., 1994). The biphasic response may be an indication of high and low affinity GABA sites that exert differential effects on the chloride pore (Pregenzer et al., 1993; Im et al., 1994). Im et al. (1994) concluded that adding the γ2 subunit seemed to abolish or reduce coupling between high affinity GABA sites and TBPS sites, and also changed coupling between the low affinity GABA sites and TBPS sites, resulting in lower inhibitory affinities (higher IC_{50}) in α1β2γ2 receptors. We found GABA to stimulate [^{3}H] EBOB binding in α1β2γ2 receptors to a maximum of 250% of control at 10 μM, a higher stimulation at about a 10-fold higher concentration than with [^{35}S] TBPS from the study by Im et al (1994). Our inhibitory IC_{50} value was 120 μM, roughly 17-fold higher than that obtained by Pregenzer et al. (1993) with [^{35}S] TBPS in α1β2γ2 receptors. Whether or not the presence of the γ2 subunit affects GABA modulation of [^{3}H] EBOB binding in the same way as [^{35}S] TBPS binding remains to be determined.

Functional Inhibition of GABA-Mediated Chloride Current by TBOB

Even though we determined from our binding studies that TBOB and EBOB likely bind to the noncompetitive convulsant site, we wanted to examine if TBOB functionally inhibited, stimulated, or had no effect on GABA-induced current. Other functional studies have reported compounds such as avermectin and γ-butyrolactones to bind to the noncompetitive site or other sites in the channel pore, and to enhance or have no effect on GABA-induced current by acting as mixed antagonists/inverse agonists or inert antagonists (Holland et al., 1990; Huang and Casida, 1997b; Williams et al., 1997). Our whole cell patch clamp studies in human α1β2γ2 receptors showed that TBOB functionally inhibited 10 μM GABA-mediated chloride current with an IC_{50} of roughly 0.2 μM, in agreement with TBOB's affinity of 0.09 μM from competition studies with [^{3}H] EBOB (Figure 7). Stimulation of 111% of control was seen at 0.01 μM TBOB. This stimulation at low concentrations was also observed with other ligands that bind to the noncompetitive convulsant site (Narahashi et al., 1998; Nagata et al., 1994; Holland et al., 1990; Bell-Horner et al., in press).

The relationship between [^{3}H] EBOB binding and functional activity was examined by measuring the GABA-stimulated $^{36}Cl^-$ influx in cultured cerebellar granule neurons (Huang and Casida, 1996). The IC_{50} value for EBOB to inhibit 40 μM GABA-stimulated $^{36}Cl^-$ influx was 290 nM, very comparable to our functional TBOB value of 0.2 μM. The same channel blockers were tested both functionally and by competition with [^{3}H] EBOB. Both functional and binding inhibitory affinities correlated very well linearly with a correlation coefficient of 0.92 (Huang and Casida, 1996). This was the first study to look at the functional role of EBOB. Another study examined functional inhibition of 100 μM GABA-stimulated $^{36}Cl^-$ uptake by TBOB in membrane vesicles from rat cerebral cortex (Obata et al., 1988). An IC_{50} functional value of 100 nM was obtained, consistent with our functional result.

Reasons for Differences in Results

Differences in our results compared to those of the literature may have been due to a number of reasons. [^{35}S] TBPS binding was found to be strongly ion, temperature, and pH dependent (Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Gallo et al., 1985; Garrett et al, 1989; Slaney et al., 1995). The authors found KBr, temperatures near 20°C (almost no specific binding at 0°), and a pH of 7.5-8.5 to result in maximal TBPS binding. B_{max} was found to be independent of both ion species and concentration, but the receptor affinity of [^{35}S] TBPS binding increased with increasing ion concentration (Garrett et al., 1989). [^{3}H] TBOB binding was identical to that of [^{35}S] TBPS in rat brain membranes with respect to pH dependence, anion specificity, regional distribution of binding sites, and inhibition by other trioxabicyclooctanes and small cage compounds, but not with respect to temperature or salt dependence (Lawrence et al., 1985). A species difference between human and rat was seen in the distribution of some [^{3}H] EBOB binding sites (Kume and Albin, 1994). In human cerebellar cortex, higher levels of binding were seen in the granule cell layer and lower levels in the molecular layer, unlike rat cerebellum, which always had a higher level of binding in the molecular layer than in the granule cell layer. This might indicate that the α6 subunit in humans differs pharmacologically from that of the rat, that receptors containing α6 subunits may not be as abundant in the human as in the rat granule layer, or that the subtype combinations formed in the granule cell layer of the two different species may differ.

Hawkinson and Casida (1992) mentioned how important it was to use a high salt concentration (e.g., KBr) and to remove endogenous GABA to get optimal binding and first-order dissociation kinetics of [^{3}H] EBOB, [^{3}H] TBOB, and [^{35}S] TBPS in native brain tissue. Differences in our results compared to that of the literature and differences in results between the literature could have been due to brain regional differences, species difference and age, using a

lower salt concentration and/or a different salt (for example, 200 mM NaCl instead of 500 mM KBr), not washing out endogenous GABA well enough, and/or using bicuculline to increase [^{3}H] EBOB binding. It should be noted that the use of bicuculline in our cerebellar assays may have increased the number of binding sites for [^{3}H] EBOB and/or changed the K_d. Control experiments without bicuculline should have been performed to determine its effect on B_{max} and binding affinity of [^{3}H] EBOB in rat cerebellum.

Do All Noncompetitive Convulsants Bind to the Same Site?

Our data indicated that [^{3}H] EBOB binds to the same site as other noncompetitive convulsants and that some aspects of its binding are subunit dependent. Competition of [^{3}H] EBOB binding by noncompetitive convulsant drugs revealed concentration dependent inhibition at one site. Concentration dependent inhibition studies of the radiolabelled trioxabicyclooctanes by noncompetitive convulsant compounds have generally revealed competition at one site, indicating that these drugs bind to an identical or closely coupled site (Squires et al., 1983; Ramanjaneyulu and Ticku, 1984; Hawkinson and Casida, 1992; Kume and Albin, 1994; Huang and Casida, 1996; Vale et al., 1997). Not only have competition studies indicated competitive interaction at the TBPS site, but saturation studies in the presence of a noncompetitive convulsant drug presumed to bind to the same site have indicated this by an increase in K_d without a change in B_{max} (Lawrence et al., 1985; Pomes et al., 1993; Kume and Albin, 1994).

Some studies have suggested that the noncompetitive convulsant binding site may not be common for all compounds (Sakurai, 1994; Tehrani et al., 1985; Van Rijn et al., 1990). A poor correlation in the regional distribution of [^{3}H] TBOB binding compared to that of [^{35}S] TBPS was found (Sakurai, 1994). Quantitative differences existed in binding site distribution even though the qualitative patterns were similar, suggesting the possibility that the noncompetitive convulsant site may not be identical for TBOB, TBPS, or other convulsant drugs (Sakurai, 1994). Similar

qualitative patterns may indicate that the binding sites are in the same vicinity or have overlapping domains. Tehrani et al. (1985) have shown multiple TBPS binding sites with both equilibrium and displacement studies. If their data were fitted for low TBPS concentrations, as used in other studies, they would have obtained a linear Scatchard plot with an affinity in good agreement with other reported values (Tehrani et al., 1985). Hill slopes below unity from competition studies supported the heterogeneity of TBPS binding sites. Even though the heterogeneity could have been due to distinct, non-interacting receptors, Tehrani et al. (1995) favored the idea of multiple states of the same receptor. Saturation analyses of [^{3}H] TBOB binding in the presence of PTX resulted in decreases in both affinity and B_{max}, thus suggesting that TBOB and PTX may not bind to the same site in a simple competitive manner (Van Rijn et al., 1990).

Association kinetics of [^{3}H] EBOB, [^{3}H] TBOB, and [^{35}S] TBPS were monophasic in all tissue or receptor subtypes investigated (Hawkinson and Casida, 1992; Cole and Casida, 1992; Lawrence et al., 1985; van Rijn et al., 1990; Maksay and van Rijn, 1993; Ito and Ho, 1994; Squires et al., 1983; Giorgi et al., 1996; Slaney et al., 1995; Zezula et al., 1996). Dissociation of [^{3}H] EBOB in native tissue was monophasic, further strengthening the evidence for a single binding site (Hawkinson and Casida, 1992; Cole and Casida, 1992). In [^{3}H] TBOB and [^{35}S] TBPS studies, dissociation was monophasic (Hawkinson and Casida, 1992; Ito and Ho, 1994) or polyphasic, usually biphasic (Lawrence et al., 1985; van Rijn et al., 1990; Maksay and van Rijn, 1993; Squires et al., 1983; Giorgi et al., 1996). For example, in certain recombinant receptors, dissociation of [^{35}S] TBPS differed depending on the compound used to initiate it (Slaney et al., 1995; Zezula et al., 1996). If a high concentration of TBPS or PTX was used to initiate dissociation, then it was slow and monophasic, but if allosteric modulators of the TBPS binding site were used, then dissociation became rapid and biphasic. Polyphasic dissociation is dependent

on the compound used to initiate it, and likely indicates multiple kinetic affinity states as opposed to more than one distinct binding site (Maksay and van Rijn, 1993). These different kinetic states depend on the agent acting on the $GABA_A$ receptor, are interconvertible, and have distinct binding affinities (Maksay and van Rijn, 1993).

Two distinct binding sites have been proposed based on the trioxabicyclooctanes structure-toxicity relationship (Kume and Albin, 1994). One site is for compounds with small x-substituents like TBPS and another site for compounds with large x-moieties like EBOB or TBOB. This is consistent with the results by Kume and Albin (1994), where no correlation was found between TBPS and TBOB binding sites, but a strong correlation was found between TBOB and EBOB sites. The existence of various subtypes of PTX binding sites was also consistent with the PTX competition data, where the Hill slope was significantly greater than 1, allowing for the possibility of the existence of multiple PTX binding sites, even though positive cooperativity may have existed between identical sites (Kume and Albin, 1994). Our saturation data revealed linear Scatchard plots, and most of our competition data revealed Hill slopes close to, but just under unity, supporting the idea of a single EBOB binding site.

The *Drosophila melanogaster* mutant Rdl (resistant to dieldrin) has a point mutation of alanine to serine within the TM2 region, believed to line the chloride ion channel pore (Ffrench-Constant et al., 1993). Insects and vertebrates that show this resistance to cyclodienes also have a cross-resistance to other compounds thought to block the channel pore, such as PTX, TBPS, and lindane (Ffrench-Constant et al., 1993; Bloomquist, 1994). Only the alanine 302 to serine (A302S) substitution mutation is directly correlated with resistance in *D. melanogaster*, but in *D. simulans*, a homologous mutation of Ala to Ser or Gly will result in resistance (Cole et al., 1995). These mutations lower affinity and the number of binding sites for [^{3}H] EBOB and also reduce the potency of various channel blockers, muscimol, and GABA in their ability to inhibit [^{3}H]

EBOB binding (Cole et al., 1995). The alanine in the TM2 region may be a common residue for binding of noncompetitive convulsant drugs such as cyclodienes, PTX, lindane, and the trioxabicyclooctanes in vertebrates (Zezula et al., 1996; Aspinwall et al., 1997).

Our lab has found that sensitivity to PTX and dieldrin, but not PTZ, was enhanced in receptors lacking an α subunit, e.g., β2γ2 receptors (unpublished observations; Bell-Horner, in press; Bell-Horner, 1999). A valine present deep in the channel pore of the TM2 region due to the α subunit may be what causes dieldrin and PTX to have diminished potencies in αβγ receptors (Bell-Horner, 1999). The βγ receptor lacks this valine residue and has an increased number of alanines. These alanines, which are due to the β subunit, are at the same position, thus increasing the potency of dieldrin and PTX (Bell-Horner, 1999). Mutation studies in our lab of a valine to alanine in the α subunit of the α1β2 receptor have also resulted in enhanced PTX sensitivity. This binding residue should be noted with respect to the Rdl mutant that has a mutated alanine and can no longer be inhibited by dieldrin and other compounds. PTZ not sharing this property indicates that it may not bind to the same site as the other drugs, but rather to a distinct site that may have overlapping domains. The β subunit may be more critical in the binding of the drug, while the α may be more involved in modulating the affinity with which the drug binds.

Utility of [^{3}H] EBOB

Various considerations should be taken into account when choosing one radioligand over another. We believe [^{3}H] EBOB to be a better choice than [^{35}S] TBPS in radioligand binding assays because of its higher affinity and lower price. Even though [^{3}H] EBOB has a much longer half-life than [^{35}S] TBPS, chemical stability over a period of time needs to be considered. Even though [^{35}S] TBPS has a binding affinity roughly 10-fold lower, its higher specific activity and higher energy might make it more suitable than [^{3}H] EBOB for some applications. In addition, a

downside to [^{3}H] EBOB that [^{35}S] TBPS does not share is the problem with [^{3}H] EBOB sticking to certain plastics, namely polystyrene and to a lesser extent, polypropylene, thus requiring these plastics to be siliconized.

Summary and Conclusions

We have shown that the competition by noncompetitive convulsants of [^{3}H] EBOB is comparable to that of [^{35}S] TBPS, the prototypical radioligand used to study the noncompetitive convulsant site and its modulation by allosteric compounds, including therapeutics. We have successfully demonstrated that [^{3}H] EBOB is a highly useful ligand to study both native and recombinant mammalian $GABA_A$ receptors. In addition, [^{3}H] EBOB should be preferred to [^{35}S] TBPS because of its higher affinity. From our competition studies, we found that [^{3}H] EBOB binds to the same site as other noncompetitive convulsants or to a region in the channel pore sharing overlapping domains with this site. Some subunit dependent differences were found in [^{3}H] EBOB binding, notably between $\alpha1/2\beta2\gamma2$ and cerebellum or $\alpha6\beta2\gamma2$ receptors. Like [^{35}S] TBPS, [^{3}H] EBOB was also biphasically modulated by GABA in $\alpha1\beta2\gamma2$ receptors. TBOB functionally inhibited GABA-mediated current in $\alpha1\beta2\gamma2$ receptors, with a functional inhibitory affinity correlating well to that from binding studies.

One goal of the present experiments was to access differences in subunit-dependent binding of EBOB based on α subtype. Additional studies should be performed to further investigate α subunit differences, along with β and γ in different $GABA_A$ receptor combinations. GABA, along with other allosteric modulators of [^{3}H] EBOB binding, should also be examined in varying subtype combinations. [^{3}H] EBOB, like [^{35}S] TBPS, can also be used to study pharmacological properties of therapeutics acting on $GABA_A$ receptors. In addition, because [^{3}H] EBOB has a higher affinity than [^{35}S] TBPS in insects, it can be used to selectively study insecticides.

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