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Research Report

Characterization of the binding of [³H]CGP54626 to GABA_B receptors in the male bullfrog (*Rana catesbeiana*)

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ABSTRACT

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the vertebrate brain. GABA activates both ionotropic (GABA_A) and metabotropic (GABA_B) receptors in mammals. Whether non-mammalian vertebrates possess receptors with similar characteristics is not well understood. We used a mammalian GABA_B-specific antagonist to determine the pharmacology of putative receptors in the brain of an anuran amphibian, the male bullfrog (*Rana catesbeiana*). Receptor binding assays with the antagonist [³H]CGP54626 revealed a single class of high affinity binding sites (with a K_D of 2.97 nM and a B_{max} of 2619 fmol/mg protein). Binding was time- and temperature-dependent, saturable and specific. Specific binding of [³H]CGP54626 was inhibited by several mammalian GABA_B receptor agonists and antagonists. The rank order potency of agonists was: GABA = SKF97541 > (R)-Baclofen > 3-APPA. The rank order for antagonists was: CGP54626 = CGP55845 > CGP52432 > CGP35348. The GABA_A receptor ligands muscimol and SR95531 had very low affinity for [³H]CGP54626 binding sites, while bicuculline compounds had no affinity. Binding of GABA was positively modulated by CGP7930. Taurine did not allosterically modulate GABA binding but did inhibit [³H]CGP54626 binding in a linear fashion. Bullfrog brain thus possesses binding sites with significant similarity to mammalian GABA_B receptors. These receptors differ from mammalian receptors, however, in dissociation kinetics, ligand specificity and allosteric modulation.

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1. Introduction

The inhibitory neurotransmitter γ -aminobutyric acid (GABA) is distributed at high concentrations across the vertebrate central nervous system (CNS; Ottersen and Storm-Mathisen, 1984; Hollis and Boyd, 2005; Anglade et al., 1999; Domenici et al., 1988). Mammalian receptors for GABA occur in two subclasses (Simeone et al., 2003). The GABA_A receptor subclasses contain ionotropic receptors which are pentameric

complexes forming a chloride channel (Rudolph et al., 2001). Mammalian GABA_B receptors, on the other hand, are heterodimers acting primarily via G-protein-dependent mechanisms (Couve et al., 2000; Bettler et al., 2004). Whether non-mammalian vertebrates possess GABA receptors with similar structures and mechanisms of action is unclear.

Scattered reports in fish, reptiles and birds suggest the presence of GABA_B receptors in these groups, but the evidence is indirect. In one bird species, the pigeon (*Columba livia*), the

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distribution of putative GABA_B receptors has been described using [³H]GABA as the ligand and blocking GABA_A receptors with isoguvacine (Veenman et al., 1994; Albin et al., 1991). The mammalian GABA_B agonist, baclofen, also influences synaptic activity in an avian auditory nucleus and the hippocampus (Brenowitz and Trussell, 2001; Brenowitz et al., 1998; Margrie et al., 2000). For reptiles, only turtles have been investigated. With the same procedure used in the pigeon brain, the distribution of GABA_B receptors in the turtle cerebellum has been inferred (Albin and Gilman, 1992). In addition, baclofen (or the related analogs, saclofen or phaclofen) alters the activity of neurons in the turtle olfactory bulb, spinal cord or cerebrum (Blanton and Kriegstein, 1992; Wachowiak and Cohen, 1999; Russo et al., 1998; Delgado-Lezama et al., 2004). In bony fish, baclofen influences GABAergic neurotransmission in the retina of carp and catfish and also in the electrosensory lateral line lobe of an electric fish (Berman and Maler, 1998; Lee and Bai, 2003; Bindokas and Ishida, 1991). Finally, in the lamprey spinal cord, baclofen and other mammalian GABA_B analogs alter the activation of locomotor networks (Alford and Grillner, 1991; Bussieres and El Manira, 1999; Tegner and Grillner, 2000). Overall, these studies suggest the presence of GABA_B receptors in non-mammalian vertebrates. However, this conclusion rests on the assumption that non-mammalian receptors have similar affinities for analogs, such as isoguvacine and baclofen, as the mammalian GABA_B receptors. The pharmacology of putative GABA_B receptors is not known for any of these classes of vertebrates.

For amphibians, specifically, several aspects of the GABAergic system appear highly conserved, when compared to mammals. In the bullfrog *Rana catesbeiana*, GABA itself is broadly distributed across the brain (Hollis and Boyd, 2005). The location of GABA-immunoreactive cells in this species, and other amphibians, is very similar to the location in mammal brains (Hollis and Boyd, 2005; Franzoni and Morino, 1989; Naujoks-Manteuffel et al., 1994; Simmons and Chapman, 2002; Barale et al., 1996). In addition, the GABA_A receptor subtype of the bullfrog is also substantially similar to the mammalian receptors in this family. This similarity includes the subunit structure, ligand binding characteristics and modulation by neurosteroids (Hollis and Boyd, 2003; Hollis et al., 2004). Conservation of the GABA_A receptor in amphibians suggests that the same might be true for GABA_B receptors.

Indirect evidence supports the hypothesis that amphibian brain contains a GABA_B-like receptor. Conservation of a GABA_B receptor antagonist binding site across several vertebrate classes (including a frog; species not reported) was shown by photoaffinity labeling of putative receptors with the analog CGP71872 (Kaupmann et al., 1997). In addition, a mammalian GABA_B-receptor-specific antibody labels cells in the bullfrog retina (Zhang and Yang, 1999). In neither of these cases, however, were any characteristics of the frog receptors determined. The presence of GABA_B receptors in amphibians is further supported by studies using mammalian GABA_B agonists and antagonists. This conclusion is strongest in the retina. In the salamander retina (*Ambystoma tigrinum* or *Necturus maculosus*), ganglion cells and amacrine cells respond to multiple GABA_B analogs, including some of the newer Ciba-Geigy analogs (Tian and Slaughter, 1994; Awatramani et al., 2001; Shen and Slaughter, 1999, 2001; Zhang et al., 1997).

Importantly, the potency of these analogs is not identical to potency in mammals (e.g., Tian and Slaughter, 1994) which suggests differences between species in GABA_B receptor pharmacology. The retina of frogs (*Xenopus laevis* and *R. catesbeiana*) shows similar responses to GABA_B analogs, compared to salamander retina (Yang, 2004; Boatright et al., 1994; Arnarsson and Eysteinnsson, 1997). Electrophysiological responses to GABA_B analogs also occur in other frog tissues, including the dorsal root ganglion (Xi et al., 1997), the spinal cord (Ovsepyan and Veselkin, 2003; Wall and Dale, 1993; Kudo et al., 1981a,b; Padjen and Mitsoglou, 1990), the cerebellum (Strauss, 1986) and the optic tectum (Sivilotti and Nistri, 1988). Finally, the amphibian olfactory bulb likely contains GABA_B receptors. Bulb field potentials in both a salamander and a frog are influenced by baclofen (Cinelli and Salzberg, 1992; Potapov and Trepakov, 1986). More directly, electrophysiological recordings in mitral cells of *Rana ridibunda* show specific responses to baclofen and saclofen (Duchamp-Viret et al., 2000). Interestingly, the amino acid taurine interacts with saclofen in the frog olfactory bulb (Chaput et al., 2004). The mechanism for this interaction is unclear. Overall, the physiological responses of frog neurons to mammalian GABA_B analogs support the hypothesis that amphibians possess functional GABA_B receptors. The location and pharmacologic properties of these receptors are not known.

The hypothesis to be tested was that the amphibian CNS contained specific binding sites with pharmacology appropriate for a member of the GABA_B receptor family. We used [³H]CGP54626 as the ligand to characterize putative receptors from bullfrog brain membrane fractions. This ligand acts as a GABA_B-receptor-specific antagonist in rat brain and has been used for the most extensive characterization of rat CNS receptors (Brugger et al., 1993; Bischoff et al., 1999). We thus determined the number, affinity, kinetics and ligand specificity of [³H]CGP54626 binding sites in the bullfrog CNS. In addition, we tested the interaction of bullfrog binding sites with the mammalian GABA_B allosteric modulator, CGP7930 (Urwyler et al., 2001). Rat GABA_B receptor binding is positively modulated by this compound so we would expect the same in bullfrogs, if binding sites show significant structural homology. Finally, we tested the interaction of taurine with bullfrog [³H]CGP54626 binding sites. Taurine interacts with rodent brain GABA_B receptors, under some conditions (see Discussion), and has electrophysiological effects in frog olfactory bulb, as discussed above.

2. Results

Bullfrog brains possess putative receptors with pharmacology substantially similar to mammalian GABA_B receptors. Membranes from adult males had binding sites for the mammalian GABA_B-specific antagonist, [³H]CGP54626. The binding of this analog was saturable, showed appropriate kinetics, was tissue- and ligand-specific and could be allosterically modulated. Specifically, binding of [³H]CGP54626 was time- and temperature-dependent. Association was rapid at room temperature (22 °C), achieving equilibrium within 4 min ($T_{1/2} = 1.3$ min; Fig. 1A). Association was slower on ice (4 °C), reaching equilibrium within 90 min ($T_{1/2} = 23$ min; Fig. 1A).

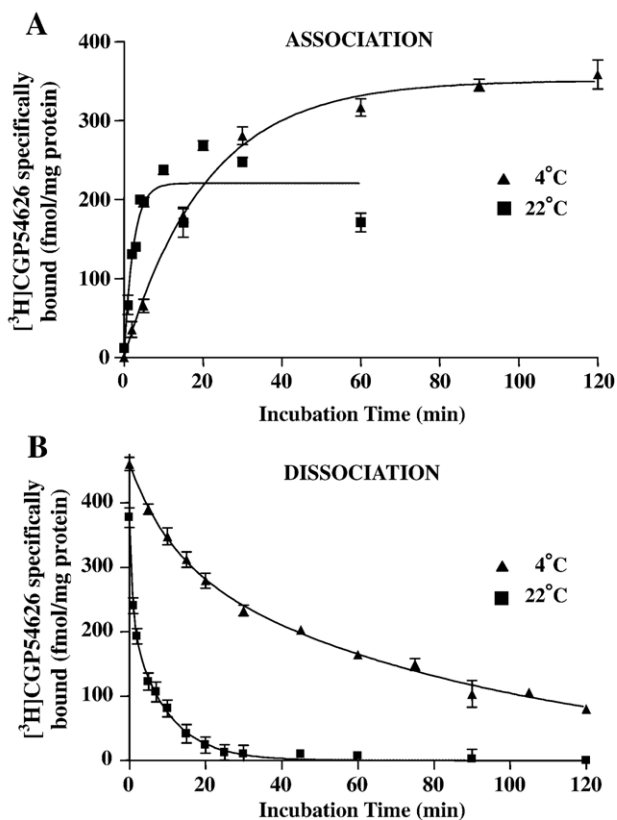


Fig. 1 – Kinetics of $[^3\text{H}]\text{CGP54626}$ binding in bullfrog brain tissue. (A) Association of 1 nM $[^3\text{H}]\text{CGP54626}$ binding to membranes incubated at 4 °C (triangles) and at 22 °C (squares). (B) Dissociation of 1 nM $[^3\text{H}]\text{CGP54626}$ binding by the addition of 1 mM GABA after 90 min association at 4 °C (triangles) and at 22 °C (squares).

The association rate constants (k_{11}) for 22 °C and 4 °C incubations were 0.4235 and 0.0466 $\text{nM}^{-1} \text{min}^{-1}$, respectively. Dissociation experiments at 22 °C showed that specifically bound $[^3\text{H}]\text{CGP54626}$ fell to less than 10% of maximum specific binding after 20 min ($T_{1/2} = 3.3$ min; Fig. 1B). After 120 min, the 4 °C dissociation experiments still had greater than 15% of specifically bound $[^3\text{H}]\text{CGP54626}$ remaining ($T_{1/2} = 25$ min; Fig. 1B). Nonlinear regression analysis comparing a one-phase versus a two-phase exponential decay model demonstrated that a two-phase model was significantly better at describing the results (F test; $P < 0.0001$). The dissociation rate constants for the 22 °C experiments were 0.1068 and 1.253 min^{-1} . The dissociation rate constants for the 4 °C experiments were 0.01138 and 0.09108 min^{-1} .

Increasing concentrations of $[^3\text{H}]\text{CGP54626}$ led to saturation of the binding sites (Fig. 2). Saturation occurred at a concentration of 10–20 nM free ligand. Nonlinear regression showed that the receptor had one binding site with high affinity ($K_D = 2.97$ nM) and low capacity within the brain ($B_{\text{max}} = 2619$ fmol/mg protein).

Agonists and antagonists of mammalian GABA_B receptors potentially displaced $[^3\text{H}]\text{CGP54626}$ bound to the bullfrog brain membranes. The rank order of GABA_B receptor antagonist potency was $\text{CGP54626} = \text{CGP55845} > \text{CGP52432} > \text{CGP35348}$

(Fig. 3A, Table 1). The rank order of GABA_B receptor agonist potency was $\text{GABA} = \text{SKF97541} > (\text{R})\text{-Baclofen} > 3\text{-APPA}$ (Fig. 3B, Table 1). The GABA_A-receptor-specific ligands SR95531 and muscimol displaced $[^3\text{H}]\text{CGP54626}$ at high concentrations (Fig. 3C, Table 1). The GABA_A-receptor-specific antagonists bicuculline and bicuculline methiodide did not displace $[^3\text{H}]\text{CGP54626}$ from the bullfrog brain membranes (Fig. 3C).

The synthetic compound CGP7930 allosterically modulated the bullfrog GABA_B-like receptor. Displacement of $[^3\text{H}]\text{CGP54626}$ by GABA was best described by a two-site binding model (F test: $F = 6.3$, $P < 0.006$), when CGP7930 (30 μM) was present. Thus, analysis revealed a high affinity ($\text{EC}_{50} = 3.3 \times 10^{-7}$) and a low affinity ($\text{EC}_{50} = 7.5 \times 10^{-6}$) component of GABA binding (Fig. 4). CGP7930 had no effect on the saturation binding isotherm for $[^3\text{H}]\text{CGP54626}$ (Fig. 4, inset; t test, $P = 0.87$). Control experiments showed no effect of CGP7930 on binding of $[^3\text{H}]\text{CGP54626}$ at several different concentrations (1–30 μM ; data not shown; t test, $P = 0.9$).

The amino acid taurine inhibited specific binding of $[^3\text{H}]\text{CGP54626}$ in a linear fashion (Fig. 5; ANOVA, $F = 5.27$, $P < 0.003$; post-test for linear trend, $P < 0.004$). Taurine did not allosterically modulate binding of GABA (Fig. 5, inset).

The $[^3\text{H}]\text{CGP54626}$ binding site was limited in its distribution outside the bullfrog brain. High levels of binding were found in the retina, with there was little binding in the liver, testes, kidney and bladder (Fig. 6).

3. Discussion

GABAergic neurotransmission in the bullfrog brain likely involves putative GABA_B subtype receptors. Using the GABA_B-specific ligand $[^3\text{H}]\text{CGP54626}$, we have identified binding sites with a pharmacology appropriate to a member of the GABA_B family. These binding sites met several requirements for classification as bona fide receptors, including tissue and ligand specificity, time and temperature dependence of kinetics and presence at high affinity and in limited capacity. This is the first systematic description of the pharmacology of the GABA_B receptor in any non-mammalian vertebrate. The

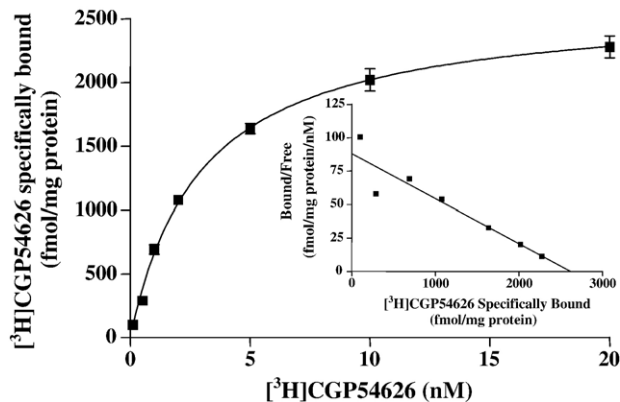


Fig. 2 – Affinity and capacity of bullfrog brain binding sites for $[^3\text{H}]\text{CGP54626}$. Saturation binding isotherm for $[^3\text{H}]\text{CGP54626}$ binding in membranes. (Inset) Scatchard transformation of saturation isotherm data.

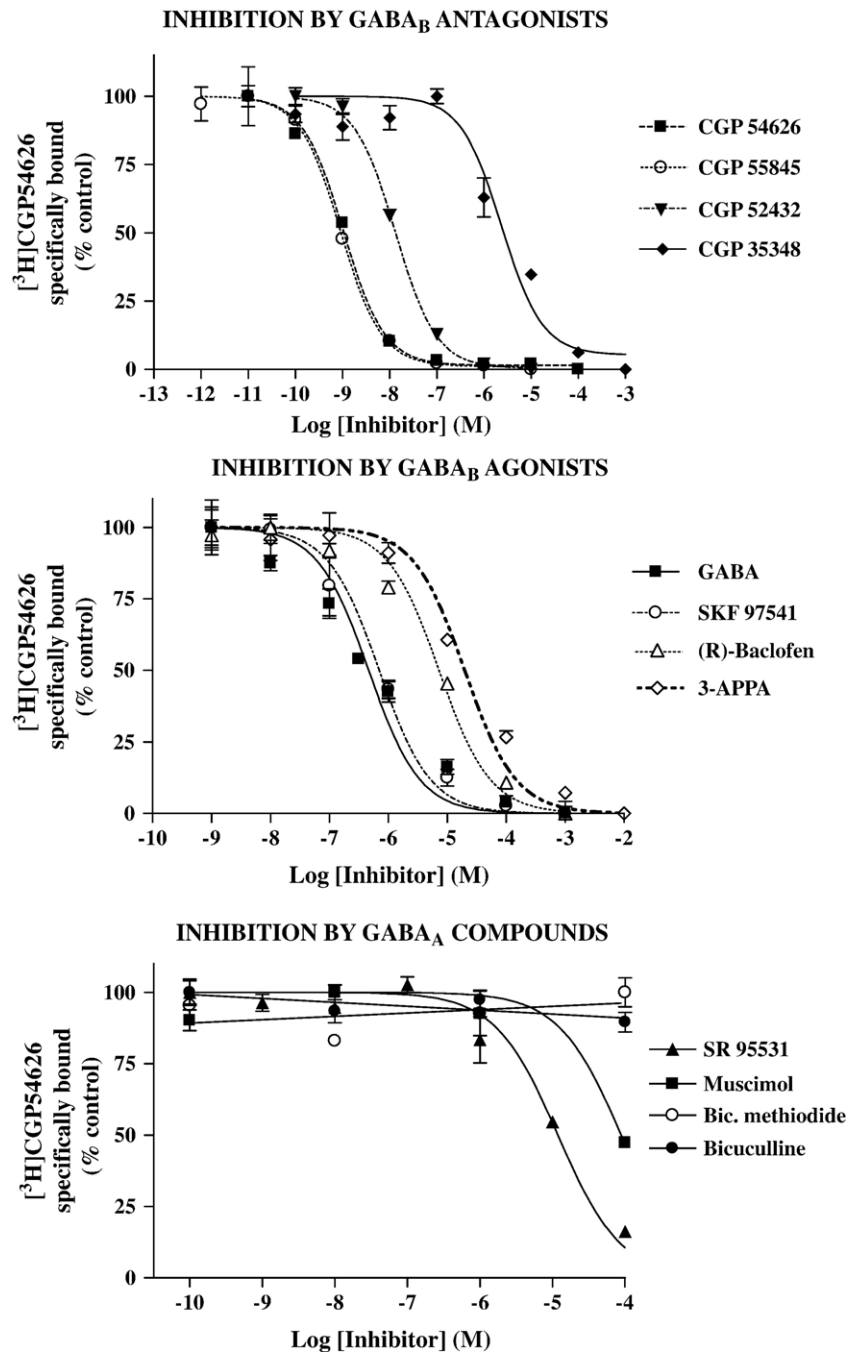


Fig. 3 – Ligand specificity of [³H]CGP54626 binding sites in bullfrog brain. Inhibition of 1 nM [³H]CGP54626 binding in bullfrog brain membranes by (A) mammalian GABA_B receptor antagonists, (B) mammalian GABA_B receptor agonists and (C) mammalian GABA_A-receptor-specific compounds.

receptor was substantially similar to mammalian receptors. Important similarities included association kinetics, K_D , B_{max} , positive modulation by CGP7930 and inhibition of binding by various classes of GABA_A and GABA_B analogs. The bullfrog binding sites did differ from mammalian sites in some minor regards, including dissociation kinetics, rank order of ligand specificity and interaction of CGP7930 with [³H]CGP54626 in saturation binding assays.

Kinetics of the bullfrog brain [³H]CGP54626 binding sites were similar to those of the rat, under comparable conditions.

At room temperature, we found $T_{1/2}$ times of 1.3 and 3.3 min for association and dissociation, respectively. This is comparable to the rates of association ($T_{1/2} = 1.5$ min) and dissociation ($T_{1/2} = 5.3$ min) in rat brain membranes, also using [³H]CGP54626 as the labeled ligand (Bischoff et al., 1999). The kinetics of [³H]CGP54626 binding at 4 °C in bullfrog brains were, on the other hand, quite different from the only previous report of binding to GABA_B receptors at this temperature. Chu et al. (1990) used quantitative autoradiography with [³H]GABA, and blocked GABA_A receptors with isoguvacine, in rat brains.

Table 1 – Inhibition of [³H]CGP54626 binding to male bullfrog brain membranes by GABA analogs

Compound	Bullfrog EC ₅₀ (μM)	95% Confidence interval (μM)	Rat EC ₅₀ (μM) ^a
GABAB receptor antagonists			
CGP54626	0.001	0.0009–0.0014	0.29
CGP55845	0.001	0.0008–0.0011	
CGP52432	0.014	0.012–0.016	0.08
CGP35348	2.98	1.36–6.53	18
GABAB receptor agonists			
GABA	0.465	0.27–0.81	0.31
SKF 97541	0.705	0.48–1.03	
(R)-Baclofen	7.28	4.59–11.54	0.35
3-APPA	19.81	12.25–32.1	
GABAA receptor analogs			
SR95531	11.87	7.47–18.85	
Muscimol	88.78	37.44–210.5	

^a EC50 values for the rat from Bischoff et al. (1999).

At 4 °C, they reported a *T* 1/2 for association of 7.15 min. This is much faster than in bullfrog brain membranes where we had a *T* 1/2 for association of 23 min, at 4 °C. This discrepancy may be due to differences in ligand affinity. Alternatively, it could be caused by differences in binding site source. Our extensive washes likely removed endogenous GABA, while the tissue slices used in autoradiography might retain significant concentrations. This would allow endogenous GABA to compete with the [³H]GABA for binding sites, decreasing the number of receptors available for [³H]GABA binding and therefore decreasing the time to reach equilibrium.

Dissociation of [³H]CGP54626 from bullfrog brain binding sites revealed a feature not previously reported for any mammalian binding sites. Nonlinear regression analysis

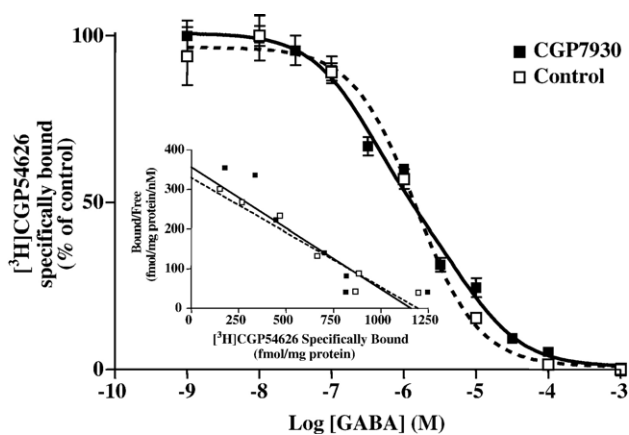


Fig. 4 – Allosteric modulation of [³H]CGP54626 in bullfrog brain. Modulation of the displacement of 1 nM [³H]CGP54626 by GABA in the presence (filled squares) or absence (open squares) of CGP7930 (30 μM). (Inset) Scatchard analysis of saturation binding isotherm in the presence or absence of CGP7930 (30 μM).

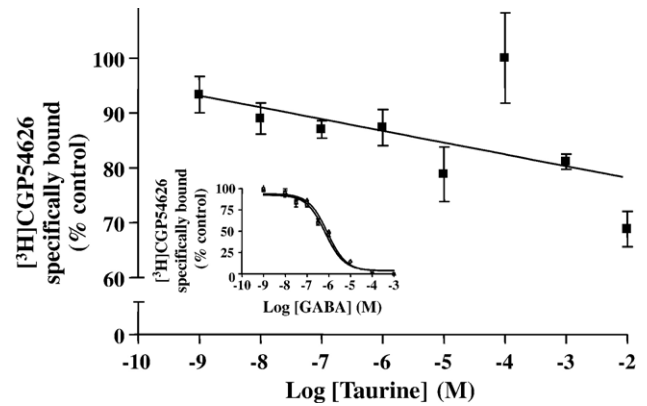


Fig. 5 – Effects of taurine on [³H]CGP54626 binding sites in bullfrog brain. Inhibition of [³H]CGP54626 binding to bullfrog brain membranes by increasing concentrations of taurine. (Inset) Inhibition of [³H]CGP54626 binding by GABA in the presence of 1 mM (open triangle), 5 mM (square) or without (open circle) taurine.

demonstrated that a two-phase exponential decay model fit the bullfrog data better than a one-phase model. A likely explanation for this phenomenon is based on current models of G-protein-coupled receptor (GPCR) activation (Hall, 2000; Parmentier et al., 2002). These models hypothesize that native receptors exist both coupled and uncoupled to G-proteins, creating two agonist affinity states. Binding of an antagonist stabilizes the receptor in the uncoupled state. GPCRs uncoupled from G-proteins have a lowered affinity for agonists. Thus, since our radioligand is an antagonist, any receptor bound to it would be stabilized in an uncoupled, lower affinity state. Addition of GABA to the system creates an ever increasing pool of receptors coupled to G-proteins and therefore in a higher affinity state. We suggest that the presence of receptor in G-protein-coupled and -uncoupled states was responsible for the dissociation of [³H]CGP54626 matching a two-phase exponential decay model.

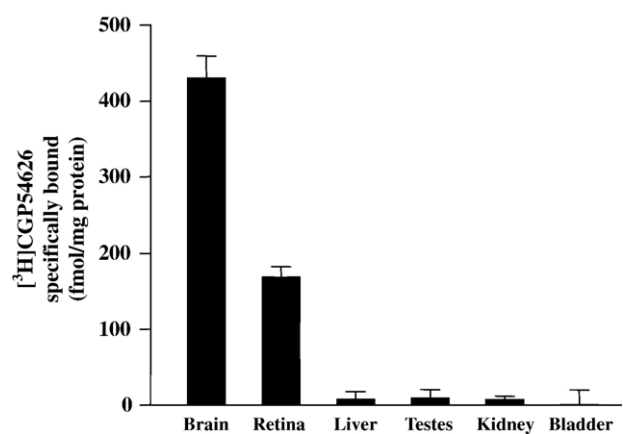


Fig. 6 – Tissue specificity of binding of 1 nM [³H]CGP54626 to membrane fractions from several bullfrog tissues. Bars are mean ± SEM of triplicates.

The affinity and number of [^3H]CGP54626 binding sites in the bullfrog brain were similar to the affinity and number of mammalian GABA_B receptors. [^3H]CGP54626 binding in bullfrog brain membranes was of high affinity ($K_D = 2.97$ nM) and comparable to that found in rat brain membranes ($K_D = 1.7$ nM, Bischoff et al., 1999; $K_D = 2.3$ nM, Hirst et al., 2003) and two human GABA_B receptor splice variants expressed in mammalian cells ($K_D = 1.51$ nM, 0.86 nM or 4.2 nM; Green et al., 2000; Hirst et al., 2003). The density of the binding sites was also similar in bullfrog brain membranes ($B_{\text{max}} = 2619$ fmol/mg protein) and rat brain membranes ($B_{\text{max}} = 3003$ or 1950 fmol/mg protein; Bischoff et al., 1999; Hirst et al., 2003). This supports the hypothesis that there is a significant structural similarity in vertebrate GABA_B receptors and suggests that the distribution of receptors across the CNS might be similar.

When [^3H]GABA is used as the labeled ligand, bullfrog brain membranes have a binding site density of 1600 fmol/mg protein (Enna and Snyder, 1977). When the GABA_A agonist [^3H]muscimol is used as the ligand, binding site density in the same species is 1800 fmol/mg protein (Hollis and Boyd, 2003). This suggests that all GABA receptors in the bullfrog brain must be GABA_A subtype receptors. However, Enna and Snyder (1977) used the standard Tris–citrate buffer. This buffer lacks the Na⁺, K⁺, Ca²⁺ and Mg²⁺ ions necessary for optimal GABA_B receptor activity (Bowery et al., 1983). For example, [^3H]GABA binding in rat brain membranes increases approximately 35% in the presence of CaCl₂ (Bowery et al., 1983). In addition, treatment with the detergent Triton X-100 greatly increases binding capacity for [^3H]GABA in the bullfrog brain (Enna and Snyder, 1977). Treatment with Triton X-100, however, reduces the binding capacity of rat GABA_B receptors for [^3H]GABA by 50% (Bowery et al., 1983). Therefore, Enna and Snyder (1977) likely significantly underestimated the total number of [^3H]GABA binding sites in the bullfrog brain. The high specificity of [^3H]muscimol for GABA_A receptors and [^3H]CGP54626 for GABA_B receptors makes them ideal ligands for determining the full capacity for GABA receptor binding in bullfrog brain. Thus, bullfrog brain possesses a greater concentration of GABA_B receptors, compared to GABA_A receptors. This is similar to rats, where there are 2–12 times as many GABA_B receptors as there are GABA_A receptors, depending on the particular brain region (Bischoff et al., 1999).

Several GABA_B receptor analogs inhibited [^3H]CGP54626 specific binding in a dose-dependent manner in bullfrogs. The rank order potency of four Ciba-Geigy (now, Novartis) antagonists was CGP54626 = CGP55845 > CGP52432 > CGP35348. The order of potency in bullfrog brain was very similar to affinities in rat brain and for human receptors expressed in CHO cells (Bischoff et al., 1999; Green et al., 2000). The first three of these compounds have affinities in the low nanomolar range and are thus considerably more specific than the earlier antagonists (e.g., phaclofen) for both mammalian and bullfrog receptors. Interestingly, the pharmacology of insect GABA_B-like receptors differs significantly. *Drosophila melanogaster* GABA_B receptors are insensitive to CGP52432 and CGP35348. They do respond to CGP55845 and CGP54626, but with much lower affinities (Mezler et al., 2001). For agonists, GABA was the most potent inhibitor followed by SKF97541, (R)-Baclofen and 3-APPA. When mammalian receptors are expressed in Chinese hamster ovary (CHO) cells or a transformed human

embryonic kidney 293 cell line, SKF97541 is a more potent agonist than GABA (Brauner-Osborne and Krosgaard-Larsen, 1999; Green et al., 2000). Our finding in bullfrogs was thus unexpected and suggests subtle differences in receptor structure.

The ligand [^3H]CGP54626 proved to be a specific analog for putative GABA_B receptors in bullfrogs. GABA_A-specific compounds did not significantly inhibit [^3H]CGP54626-specific binding in bullfrog membranes. Neither bicuculline compound displaced the radioligand. As in the rat, muscimol inhibited binding only at high concentrations (Hill and Bowery, 1981; Bowery et al., 1983). In the bullfrog, SR95531 also inhibited binding of [^3H]CGP54626 at high concentrations. Because baclofen does not inhibit binding of [^3H]SR95531 in rats, it had been previously suggested that SR95531 does not bind to GABA_B receptors (Heaulme et al., 1987). Our results suggest that SR95531 may be less specific for the GABA_A receptor than previously thought.

The bullfrog [^3H]CGP54626 binding site was positively modulated by CGP7930. The increased affinity of GABA for GABA_B receptors was demonstrated by the biphasic displacement curve in the presence of CGP7930. A similar inhibition curve was described for [^3H]CGP62349, another GABA_B antagonist, when assayed with recombinant human GABA_B heterodimers expressed in CHO cells (Urwyler et al., 2001). CGP7930 appears to act directly on the transmembrane domain (TMD) of the GABA_{B2} subunit (Binet et al., 2004). This interaction is hypothesized to stabilize the TMD of the GABA_{B2} subunit in the active state (Binet et al., 2004). The high level of allosteric interaction between the subunits of the GABA_B heterodimer (Liu et al., 2004) leads to stabilization of the TMD and extracellular domain of the GABA_{B1} subunit. The GABA_{B1} subunit thus would show a higher affinity for agonists, in the presence of CGP7930 (Binet et al., 2004). Thus, addition of CGP7930 creates a larger pool of GABA_B receptors with the GABA binding site stabilized in a high-affinity state than under natural circumstances. Results of the modulation experiment therefore suggest that CGP7930 leads to display of two affinity states of a single [^3H]CGP54626 binding site. Allosteric modulation of bullfrog GABA_B receptors supports the hypothesis that the bullfrog has a GABA_{B2} subunit with significant sequence homology to the mammalian form of the subunit.

On the other hand, CGP7930 did not increase the affinity of [^3H]CGP54626 for the bullfrog GABA_B receptor in saturation binding assays. This modulator does increase the affinity of GABA_B receptors from rat cortical membranes for [^3H]CGP27492, a GABA_B receptor agonist (Urwyler et al., 2001). Based on current models of GABA_B receptor function, this discrepancy is not unexpected. The GABA_B receptor ligand binding domain is a venus flytrap motif with two lobes. Binding of an agonist causes a conformational change in the binding domain that stabilizes the TMD in an active state (Hall, 2000; Parmentier et al., 2002). However, [^3H]CGP54626 is an antagonist. It thus prevents closure of the ligand binding domain, which stabilizes the receptor in an inactive form and alters the allosteric binding site for CGP7930 (Binet et al., 2004). By this model, CGP7930 increases affinity for agonists (such as CGP27492), but not antagonists (such as CGP54626). Therefore, we did not see an increased affinity for [^3H]CGP54626 when CGP7930 was

included in saturation binding experiments. The interaction of the bullfrog GABA_B receptor with the allosteric modulator CGP7930 thus differs from the interaction of the mammalian receptor with this analog.

Taurine is structurally similar to GABA, and it is still unclear how this amino acid modulates excitability in the CNS. In bullfrog brain membranes, taurine inhibited binding of [³H]CGP54626 at high concentrations (around 10⁻⁵ M). Whether taurine interacts with GABA_B receptors from rodent brains is unclear. Similar to our findings in the bullfrog, in early studies in mouse brain, taurine inhibited [³H]GABA and [³H]baclofen binding to GABA_B receptors with IC₅₀ values of 5.12 and 1.49 μM, respectively (Kontro and Oja, 1990; Kontro et al., 1990). In addition, there is evidence that taurine prevents GABA or glutamate release through activation of presynaptic GABA_B receptors (Kamisaki et al., 1996; Belluzzi et al., 2004). Other studies, however, find no effect of taurine on GABA_B receptor mechanisms and emphasize effects on GABA_A receptors (e.g., del Olmo et al., 2000). Both frog and rat olfactory bulb contain very high levels of taurine (Kratskin et al., 2000; Ross et al., 1995). The amino acid reduces activity of mitral cells in the bulb of both species via a presynaptic inhibition of glutamate release from olfactory receptor neurons (Belluzzi et al., 2004; Chaput et al., 2004). In frogs, there is strong evidence that this inhibition by taurine is due to a GABA_B-receptor-mediated mechanism (Chaput et al., 2004). Our study supports the hypothesis that taurine is a GABA_B receptor agonist. Lastly, it has been proposed that a variety of amino acids can act as allosteric modulators of the GABA_B receptor (Kerr and Ong, 2003). In bullfrog brains, taurine did not allosterically modulate binding of GABA to [³H]CGP54626 binding sites. This hypothesis is thus not supported for bullfrogs nor for rats (Urwylter et al., 2004).

Functional GABA_B receptors were not abundant outside the brain in bullfrogs. Retina had moderate levels of [³H]CGP54626 specific binding, but other peripheral tissues showed low levels of binding. Similarly, in rats, photoaffinity labeling shows barely detectable concentrations of functional GABA_B receptors in such tissues (Belley et al., 1999). Both GABA_{B1a} and GABA_{B1b} subunit isoforms are expressed in liver, testes, kidney and bladder of some mammals (Castelli et al., 1999; Calver et al., 2000). However, GABA_{B2} subunit expression was nearly absent in all peripheral tissues tested (Calver et al., 2000). Because heterodimerization is required for ligand binding in all species investigated so far, lack of the GABA_{B2} subunit could account for low levels of radioligand binding in mammals and bullfrogs.

GABA_B receptors are likely involved in many aspects of amphibian brain function. Strong evidence exists for the involvement of GABA_B receptors in olfaction (Potapov and Trepakov, 1986; Cinelli and Salzberg, 1992; Duchamp-Viret et al., 2000; Chaput et al., 2004) and vision (Boatright et al., 1994; Tian and Slaughter, 1994; Arnarsson and Eysteinnsson, 1997; Zhang and Yang, 1999; Zhang et al., 1997; Shen and Slaughter, 1999; Awatramani et al., 2001; Shen and Slaughter, 2001). Receptor binding assays with [³H]CGP54626 revealed that the putative bullfrog GABA_B receptor was substantially similar to the mammalian form of the receptor. However, we found subtle differences in dissociation kinetics, ligand affinity and allosteric modulation which

may indicate structural or functional differences between amphibian and mammalian receptor types. Knowledge of these differences in amphibian GABA_B receptor pharmacology will allow future hypothesis-driven experiments on the function of this neurotransmitter system in non-mammalian vertebrates.

4. Experimental procedures

Adult male bullfrogs (*R. catesbeiana*) were purchased from C. Sullivan Company (Nashville, TN) and housed in the laboratory in large tanks (50 × 21 × 21 cm) with flow-through water. Frogs were fed goldfish and kept in a 12L:12D controlled photoperiod at 17 °C. They were anesthetized on ice and then rapidly decapitated. Brains were removed and weighed immediately before homogenization. All experiments were performed in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and had been approved by the University of Notre Dame IACUC.

Membrane preparation procedures were modified from Bischoff et al. (1999). Individual brains were homogenized for 30 s with a Tissue Tearor™ in 25 volumes (2.5 ml per 100 mg tissue) of an ice-cold buffer containing 320 mM sucrose, 1 mM KH₂PO₄ and 1 mM MgCl₂. The homogenate was centrifuged at 1000×g for 15 min at 4 °C, resuspended with vigorous vortexing and the previous spin was repeated. Supernatants from two or three brains were pooled and centrifuged at 18,000×g for 15 min at 4 °C. The pellet was resuspended in 5 ml of ice-cold, distilled, deionized water, left on ice for 30 min and then centrifuged at 39,000×g for 15 min at 4 °C. Lastly, the pellet was resuspended in 25 volumes of Krebs–Henseleit (KH) buffer (120 mM NaCl, 6 mM glucose, 20 mM Tris, 4.7 mM KCl, 1.8 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, pH 7.4) and frozen at –20 °C for at least 42 h before use. On the day of the assay, the membranes were thawed at room temperature and then kept on ice. Membranes were then washed 3 times with 25 volumes of ice-cold KH buffer by centrifugation at 18,000×g for 15 min at 4 °C. The pellet was resuspended in 10 volumes of KH buffer for use in the assays. Peripheral tissues were processed in the same manner.

Receptor kinetics assays (association and dissociation) were performed in 1 ml reaction volumes containing 100 μl membrane suspension (40–120 μg total protein), and 1 nM (final concentration) [³H]CGP54626 (Tocris; ([S-(R*,R*)]-[3-[[1-(3,4-Dichlorophenyl)ethyl] amino]-2-hydroxypropyl]]([3,4-³H]-cyclohexylmethyl)phosphinic acid); 40 Ci/mmol) in KH buffer. Tubes with short incubation times (≤5 min; association and dissociation) were incubated as 3 individual 1 ml reactions. The remaining assays (association and dissociation >5 min, saturation, inhibition, modulation) were performed by combining triplicates in one incubation tube for a final reaction volume of 3 ml. Dissociation assays were performed by incubating the membranes with radioligand for 90 min to reach equilibrium. Then, 100 mM GABA dissolved in KH buffer was added to the reaction (10 μl per ml reaction solution) for a final GABA concentration of 1 mM. The saturation binding isotherm for [³H]CGP54626 was determined by incubating increasing concentrations of the radioligand with membranes for 90 min before termination of the reaction.

Ligand specificity was determined by addition of increasing concentrations of agonists and antagonists of both GABA_A and GABA_B receptors to the radioligand solution immediately before addition of membrane preparations. Reactions were terminated after 90 min. GABA_B receptor agonists tested were GABA (Sigma), SKF97541 (Tocris), (R)-baclofen (Tocris) and 3-aminopropylphosphonic acid (3-APPA; Sigma). GABA_B receptor antagonists tested were from Tocris and included CGP54626, CGP55845, CGP52432 and CGP35348. GABA_A-receptor-specific compounds tested were muscimol (Sigma), bicuculline (Sigma), bicuculline methiodide (Sigma) and SR95531 (Tocris).

Modulation experiments were performed using the same methods as the inhibition experiments but included the addition of candidate modulators (CGP7930 or taurine) before addition of the membrane solution. Specifically, 30 µl of CGP7930 (300 µM in DMSO) or taurine (100 or 500 mM in KH buffer) was added to 2700 µl of 1.11 nM [³H]CGP54626 in KH buffer and 30 µl of GABA solution (100 nM to 100 mM in KH buffer). Membrane solution in KH buffer (300 µl) was then added, and the tube was vortexed briefly to mix.

Non-specific binding for each assay was determined in the presence of 1 mM GABA. Specific binding was determined by subtracting non-specific binding from total binding. Hydrophobic compounds were dissolved in ethanol or DMSO. Control experiments showed that these solvents do not affect binding at concentrations used in our experiments (≤1%). All reactions were performed in triplicate and were incubated on ice unless otherwise indicated.

Reactions were terminated by vacuum filtration through Whatman GF/C glass fiber filters that were pre-soaked for 30 min in 0.03% polyethylenimine (V/V in dH₂O) followed by one 3-ml rinse with ice-cold KH buffer. Radioactivity was counted in 10 ml of ScintiSafe™ 30% (Fisher Scientific) for 10 min in a liquid scintillation counter after overnight incubation. Data were analyzed using GraphPad Prism (version 3.0; GraphPad Software Inc., San Diego, CA). On figures, all data points are means ± SEM of triplicates from a representative experiment. Some data points had small SEM values, and error bars are thus hidden by the symbols. Figures are representative examples of 3 or more individual experiments.

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