

Research report

# Characterization of the GABA<sub>A</sub> receptor in the brain of the adult male bullfrog, *Rana catesbeiana*

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## Abstract

Little is known about the properties of GABA receptors in the amphibian brain. The GABA<sub>A</sub> receptor is widespread in the mammalian brain, and can be specifically labeled with the receptor agonist [<sup>3</sup>H]muscimol. The binding of [<sup>3</sup>H]muscimol to membrane preparations from the brain of the bullfrog, *Rana catesbeiana*, was investigated in kinetic, saturation, and inhibition experiments to determine whether this species possessed a GABA<sub>A</sub>-like receptor. Binding of 20 nM [<sup>3</sup>H]muscimol to membranes was specific and could be displaced by 1 mM GABA. Association binding curves showed that steady state occurred rapidly, within 2 min, and dissociation occurred within 5 min. The receptor was saturable with a single, high-affinity binding site ( $K_D = 19.2$  nM;  $B_{max} = 1.8$  pmol/mg protein). Binding of [<sup>3</sup>H]muscimol was inhibited in a dose-dependent fashion by muscimol, GABA, bicuculline methiodide, and bicuculline (in order of potency). Baclofen (at doses from  $10^{-9}$  to  $10^{-3}$  M) failed to displace [<sup>3</sup>H]muscimol. The binding characteristics and ligand specificity of [<sup>3</sup>H]muscimol binding sites in the bullfrog brain support the hypothesis that this amphibian possesses a GABA<sub>A</sub>-like receptor protein similar to the GABA<sub>A</sub> receptor characterized in mammals. © 2003 Elsevier B.V. All rights reserved.

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## 1. Introduction

The amino acid neurotransmitter GABA interacts with three different families of receptors in mammalian tissues: the GABA<sub>A</sub>, GABA<sub>B</sub> and GABA<sub>C</sub> receptor families [16]. In the brain, the GABA<sub>A</sub> receptor is the most broadly distributed of these subtypes and is responsible for many diverse and important actions in the central nervous system [24,44,50,51,56,57,64]. Evidence from representative species in most vertebrate classes suggests that major elements of the GABAergic system have been conserved. For example, the avian brain also possesses receptors from the GABA<sub>A</sub> and GABA<sub>B</sub> families [6,32,36,46,55]. Binding of the mammalian GABA<sub>A</sub> receptor agonist, [<sup>3</sup>H]muscimol, occurs in most of the same regions in the quail brain as in the rat brain [15]. GABA binding to the GABA<sub>A</sub> receptor plays important roles in audition and learning and memory in birds [29,30,31,

42,45,59,63]. There are nonetheless significant differences in receptor structure between birds and mammals [10,35].

Both GABA<sub>A</sub> and GABA<sub>B</sub> receptor subtypes may be present in reptiles, but there are currently very few studies [3,33,53]. Turtle brain [<sup>3</sup>H]flunitrazepam binding and functional studies support the presence of GABA receptors with binding characteristics similar to rat receptors [17,41]. GABA and GABA analog binding suggests the presence of both high affinity GABA<sub>A</sub> receptor and GABA<sub>A</sub>/benzodiazepine receptor subtypes in an elasmobranch and teleosts [11,18,19,27,37,48,54]. The presence of similar GABA receptor families, with similar pharmacology, is thus supported in ectothermic vertebrate species studied so far. On the other hand, very little is known of the actual structure and function of GABA receptors in these classes.

In amphibians, there is pharmacological and/or immunocytochemical evidence for members of all three GABA receptor families in brain or retinal tissue. GABA<sub>A</sub>-, GABA<sub>B</sub>- and GABA<sub>C</sub>-receptor specific synthetic analogs alter the activity of cells in the retina and olfactory bulb of frogs and salamanders [21,22,23,28,40]. A GABA<sub>B</sub>-specific antibody labels cells in the frog retina [28,60]. GABA<sub>A</sub>-

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specific analog treatment alters neuroendocrine control of  $\alpha$ -melanocyte stimulating hormone, neurosteroid biosynthesis, auditory integration, and sexual behavior in amphibians [1,12,20,34,61,62]. However, none of the GABA receptor types has been well characterized in an amphibian. Furthermore, there is some evidence that GABA exerts unique effects in amphibians. For example, GABA receptor-mediated presynaptic inhibition in frog spinal cord preparations is not the same as that in rats [7,8,52]. These differences may be due to receptors with unique characteristics.

We used [ $^3$ H]muscimol, a high-affinity GABA<sub>A</sub> agonist in mammals, to characterize putative GABA<sub>A</sub>-like receptors in the adult bullfrog, *Rana catesbeiana*. Early work in bullfrogs showed binding sites for [ $^3$ H]GABA in brain and spinal cord [26]. This ligand, however, can bind to all three receptor subtypes and even to transporters. The presence of GABA<sub>A</sub>-type receptors, in particular, in frog brain is supported by three studies. First, patch-clamp studies on neurons from the optic tectum of frog (*Rana pipiens*) tadpoles have shown that bicuculline sensitive GABA receptors exert a profound effect on visual responses [38]. Second, an antibody against mammalian GABA<sub>A</sub> receptor  $\beta$ 2/3 subunits labels the brain of *R. pipiens* [4]. Third, the autoradiographic distribution of [ $^3$ H]muscimol and [ $^3$ H]flunitrazepam has been described in the frog, *Rana esculenta* [54]. However, the characteristics of [ $^3$ H]muscimol binding were not described, so it is not known whether the kinetics, affinity, concentration, or ligand specificity of frog [ $^3$ H]muscimol binding sites are similar to those sites in mammals. We used membrane preparations to characterize [ $^3$ H]muscimol binding sites in the bullfrog brain.

## 2. Materials and methods

### 2.1. Tissue and membrane receptor preparation

Adult male bullfrogs (*R. catesbeiana*) were purchased from C. Sullivan Company (Nashville, TN). Bullfrogs were housed in the lab on a 12L/12D controlled photoperiod at 17 °C in large tanks (50 × 21 × 21 cm) with flow-through water and maintained on a diet of goldfish. Bullfrogs were cryoanesthetized, rapidly decapitated and the brains removed (including brainstem, but not spinal cord) and weighed immediately before membrane preparation. 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. All chemicals and incubations were kept at 4 °C or on ice unless otherwise noted.

Receptor preparation was based on the methods of Basile [9], with modification. Brains were homogenized using a Polytron® (Brinkmann Instruments, Westbury, NY) for 12 s at approximately 1/4 full speed in 50 volumes (g brain tissue wet weight to ml of buffer) of ice-cold buffer (50mM Tris–

citrate, pH = 7.4). Two bullfrog brains were used per experiment. The homogenate was then centrifuged at 20,000 × g for 20 min. The resultant supernatant was decanted and the pellet resuspended in fresh buffer. This wash procedure was repeated an additional five times. After the final wash and removal of supernatant, the pellet was placed at –80 °C. After a minimum of 18 h, the pellet was resuspended in 50 volumes of Tris–citrate and washed two more times to yield a mixed membrane preparation with endogenous GABA removed. The mixed membrane preparation was divided into aliquots and the appropriate amount of [ $^3$ H]muscimol in buffer was added to aliquots of 750  $\mu$ l incubation volume with final protein concentrations of 0.2–1 mg/ml. All determinations within a single experiment were made in triplicate, thus each 750  $\mu$ l aliquot allowed for three replicates of 250  $\mu$ l. Membrane protein concentration was determined by the method of Bradford [13], with bovine serum albumin as standard.

### 2.2. Binding experiments

Most experiments were performed with [ $^3$ H]muscimol concentrations at 20nM (based on the methods of Tavolaro et al. [54]; specific activity = 20.0 or 28.5 Ci/mmol; NEN™, Boston, MA, USA). For association experiments, each set of triplicates was terminated (see below) after progressively longer [ $^3$ H]muscimol incubation periods. Because of the extremely rapid association of [ $^3$ H]muscimol, each 250  $\mu$ l replicate was assayed separately. For the dissociation assay, the receptor preparation was incubated with 20nM [ $^3$ H]muscimol for 20 min to reach equilibrium. After 20 min, three 250- $\mu$ l aliquots were used to determine mean total binding. Unlabeled GABA (1 mM) was then added and the reaction terminated at subsequent times. The saturation binding isotherm for [ $^3$ H]muscimol was determined following the methods of Basile [9]. Total binding was determined by incubating membranes with different concentrations of [ $^3$ H]muscimol for 90min before reactions were terminated (see below). Tissue samples from the liver, spleen, testis, and retina of bullfrogs were assayed for [ $^3$ H]muscimol specific binding at 20 nM using identical procedures as saturation binding. To determine ligand specificity, 20 nM [ $^3$ H]muscimol was added to receptor preparations with increasing concentrations of different putative GABA receptor agonists and antagonists and incubated for 20 min. Compounds included muscimol (3-hydroxy-5-aminomethyl-isoxazole; Cat. no. M1523), GABA (Cat. no. A5835), bicuculline (Cat. no. B9130), bicuculline methiodide (Cat. no. B6889), and baclofen ((+)- $\beta$ -(aminomethyl)-4-chlorobenzenepropanoic acid; Cat. no. B5399). All compounds were purchased from Sigma, St. Louis, MO, USA. Specific binding of each compound was normalized to percent of the control, which had an equal volume of buffer added. Nonspecific binding of [ $^3$ H]muscimol was determined in the presence of 1 mM unlabeled GABA. Non-specific binding was subtracted from the total binding to determine specific binding.

### 2.3. Reaction termination

All binding experiments were terminated by rapid vacuum filtration. Aliquots of 250  $\mu$ l of treated mixed membrane preparations were placed on Whatman GF/C glass microfibre filters presoaked for 15 min with 0.03% polyethylenimine in deionized, distilled H<sub>2</sub>O. Membranes on filters were then washed twice with 3 ml rinses of 50 mM ice-cold Tris–citrate. Filters were placed in vials with 10 ml of scintillation fluid (ScintiSafe™ 30%; Fisher Scientific, Pittsburgh, PA), shaken overnight, and counted on a liquid scintillation counter.

Representative results (from at least three experiments of the same type) are shown. Data were analyzed using GRAPHPAD PRISM (v. 3.0; Graph Pad, San Diego, CA, USA), which performed transformations, linear and nonlin-

ear regression, ANOVA and post hoc tests. Also, *F*-tests compared fits for one- and two-site binding models for Scatchard analysis, plus one- and two-site competition models. Linearizing inhibition data was performed to detect the possibility of multiple classes of binding sites [14].

### 3. Results

Bullfrog brain membranes possessed specific binding sites for [<sup>3</sup>H]muscimol. Specific binding of [<sup>3</sup>H]muscimol was approximately 50% of the total binding. This binding was tissue- and ligand-specific, saturable, and could be displaced by 1 mM GABA.

The kinetics of [<sup>3</sup>H]muscimol binding in the bullfrog brain showed both rapid association and dissociation (Fig. 1). The

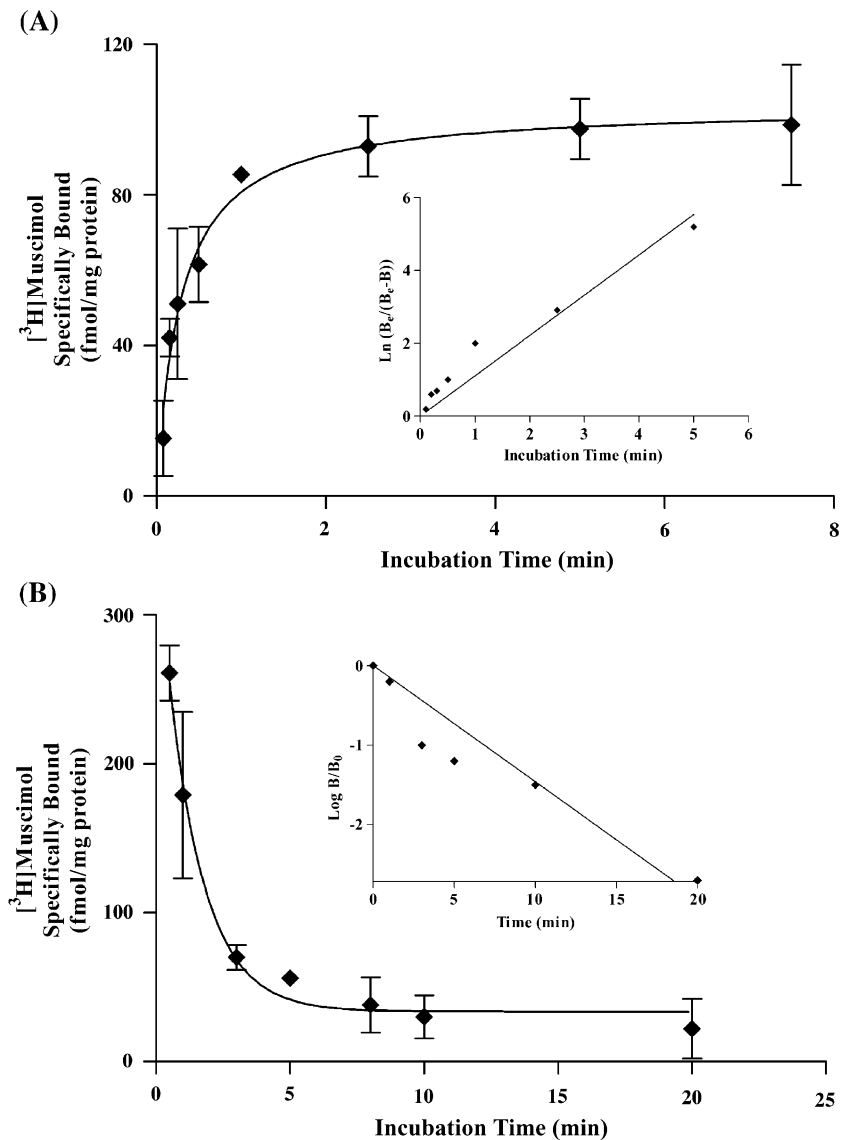


Fig. 1. Kinetics of 20 nM [<sup>3</sup>H]muscimol binding in bullfrog brain membrane homogenates (mean  $\pm$  S.E.M. of triplicates from a single representative experiment). (A) Association of [<sup>3</sup>H]muscimol at 4 °C. (Inset) Pseudo first-order association plot ( $r^2=0.91$ ). (B) Dissociation of [<sup>3</sup>H]muscimol at 4 °C with the addition of 1mM GABA following 20 min association. (Inset) Semi-logarithmic plot of dissociation ( $r^2=0.88$ ).

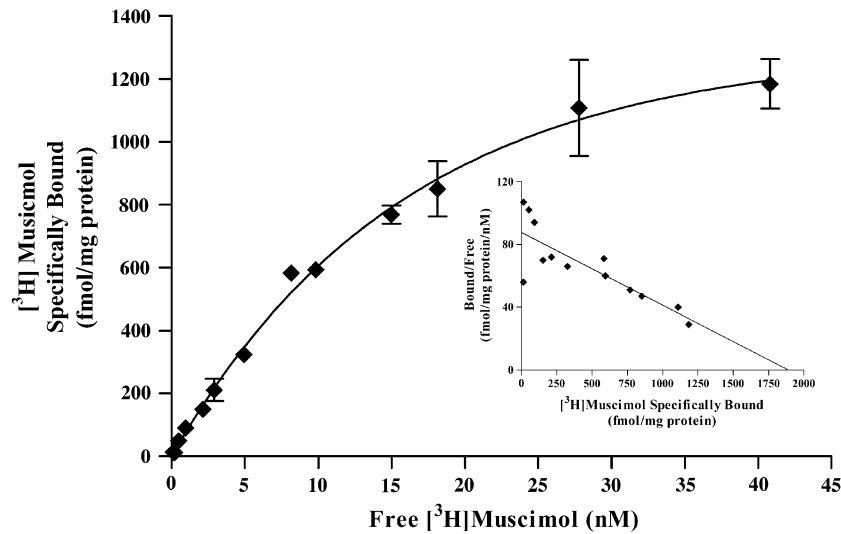


Fig. 2. Saturation binding isotherm for [ $^3\text{H}$ ]muscimol in bullfrog brain homogenates. Points are means  $\pm$  S.E.M. from triplicate determinations (some points had very small S.E.M. determinations, between 1 and 23 fmol/mg protein, and error bars are thus obscured by the symbols). (Inset) Scatchard replot of saturation data ( $r^2=0.684$ ).

association rate of specifically bound [ $^3\text{H}$ ]muscimol was the more rapid of the two, as steady state was reached in just over 2 min at 4  $^{\circ}\text{C}$  (Fig. 1A). The association rate constant ( $k_{+1}$ ) was 0.22 nM/min and the observed association was linear with log transformation (Fig. 1A, inset). The dissociation of specifically bound [ $^3\text{H}$ ]muscimol was nearly as fast as its association, with less than 10% of specific binding remaining after 5 min (Fig. 1B). The dissociation rate constant ( $k_{-1}$ ) was 0.15 nM/min (Fig. 1B, inset).

The binding of [ $^3\text{H}$ ]muscimol to bullfrog brain membranes was saturable with increased ligand concentrations (Fig. 2). Specifically bound [ $^3\text{H}$ ]muscimol increased with increasing concentrations of the radioligand and saturated at a concentration of approximately 40 nM of free radioligand

(Fig. 2). Nonlinear regression and Scatchard analysis indicated a single, high affinity binding site with a  $K_D$  of  $19.2 \pm 1.9$  nM and a  $B_{\text{max}} = 1.8$  pmol/mg protein (Fig. 2, inset).

Mammalian GABA $_A$  receptor agonists and antagonists displaced [ $^3\text{H}$ ]muscimol from bullfrog membranes in a concentration-dependent manner (Fig. 3). Unlabeled muscimol was the most potent inhibitor of [ $^3\text{H}$ ]muscimol specific binding with an  $\text{EC}_{50}$  of 0.23  $\mu\text{M}$ . GABA was the next most potent competitor, with an  $\text{EC}_{50}$  of 1.6  $\mu\text{M}$ , followed by bicuculline methiodide ( $\text{EC}_{50} = 13.2$   $\mu\text{M}$ ), and bicuculline ( $\text{EC}_{50} = 32.4$   $\mu\text{M}$ ). Baclofen, a specific mammalian GABA $_B$  receptor agonist, failed to influence [ $^3\text{H}$ ]muscimol binding at concentrations of between 10 pmol and 10  $\mu\text{M}$ .

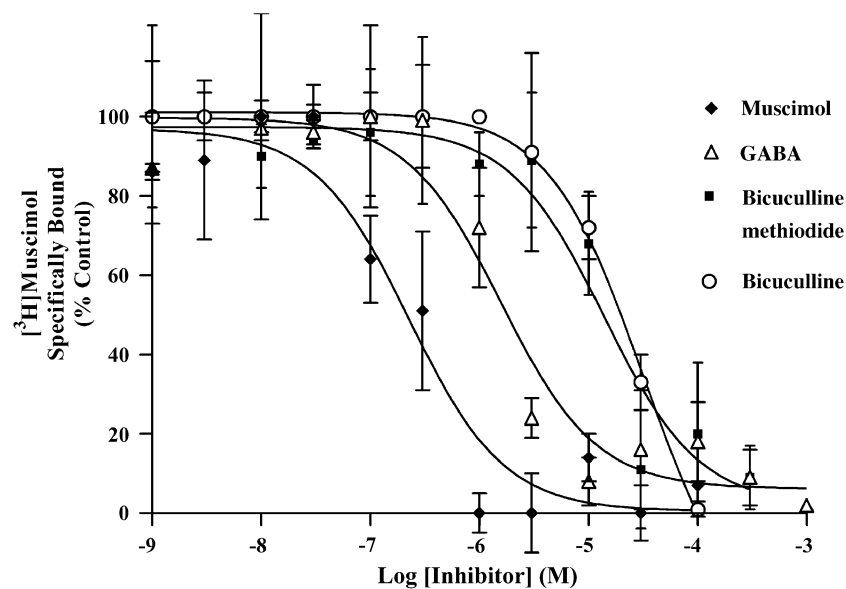


Fig. 3. Inhibition of [ $^3\text{H}$ ]muscimol binding by different chemicals. Points are mean  $\pm$  S.E.M. of triplicates.

The distribution of [ $^3\text{H}$ ]muscimol binding sites was tissue-specific. Apart from the brain, specific binding was detected in membrane preparations from the retina and testes. No specific binding was observed in membrane preparations from the liver and spleen.

#### 4. Discussion

The binding of [ $^3\text{H}$ ]muscimol to bullfrog brain membranes was tissue- and ligand-specific, time-dependent, of high affinity and of limited capacity. This supports the hypothesis that the amphibian brain possesses a GABA<sub>A</sub>-type receptor. This is the first report of kinetics, saturation binding, and ligand specificity for any GABA analog in amphibian brain.

The association rate of 20 nM [ $^3\text{H}$ ]muscimol in bullfrog membrane preparations at 4 °C was extremely rapid, reaching steady state in just over 2 min. This is very similar to [ $^3\text{H}$ ]muscimol association seen in bovine brain preparations at 25 °C [2]. Whether temperature affects [ $^3\text{H}$ ]muscimol binding is still questionable as past work has yielded contrasting results [2,58]. Dissociation of [ $^3\text{H}$ ]muscimol in the presence of unlabeled GABA was also quite rapid. This short displacement time (<5 min) is also found in the mammalian brain, in the presence of excess unlabeled GABA or muscimol [2,51].

The affinity of the bullfrog [ $^3\text{H}$ ]muscimol binding site was similar to that of other GABA<sub>A</sub> receptors. Binding of [ $^3\text{H}$ ]muscimol in bullfrog brain preparations revealed a single, high affinity binding site with a  $K_D$  of 19.2 nM, which is in the range considered to indicate a high affinity site [43]. This  $K_D$  is consistent with muscimol's greater affinity for the GABA<sub>A</sub> receptor than GABA, as seen in mammals [5]. The binding affinity of [ $^3\text{H}$ ]GABA in bullfrog brain (58 nM) and spinal cord (33 nM) membrane preparations is lower [26]. The high affinity binding of [ $^3\text{H}$ ]muscimol in bullfrogs is similar to the affinity in rat (13 and 42 nM), bovine (*Bos taurus*; 10 nM), and codfish (*Gadus morrhua*; 13.5 nM) brain [2,9,19,51]. Thus, the high affinity binding site of the GABA<sub>A</sub> receptor appears conserved in vertebrate evolution.

Binding of [ $^3\text{H}$ ]muscimol to one versus two classes of binding sites varies with species and assay conditions. Our procedure reveals high and low affinity [ $^3\text{H}$ ]muscimol binding sites in rat membrane preparations [9]. Visual examination of the Scatchard replot (Fig. 2, inset) suggests that bullfrogs might also have two binding sites. However, the presence of only a single, high affinity binding site was determined using computer assisted nonlinear regression analysis [47]. Only one class of binding site was also found in bullfrog brain when [ $^3\text{H}$ ]GABA was used as the ligand [26]. One high-affinity binding site for [ $^3\text{H}$ ]muscimol is present in codfish [19], as well as rat under some assay conditions [51]. Two classes of [ $^3\text{H}$ ]muscimol or [ $^3\text{H}$ ]GABA binding sites are more typically found in rat, bovine and

salmon brain membranes [2,9,25,27]. Although the saturation binding isotherm for bullfrogs definitively showed only one class of [ $^3\text{H}$ ]muscimol binding site, there were two indirect indications that a second (lower affinity) binding site may exist. First, the  $\text{EC}_{50}$  of muscimol in inhibition experiments was about an order of magnitude higher than expected, when compared to the affinity of [ $^3\text{H}$ ]muscimol from saturation experiments. A likely explanation is the presence of a low affinity site not detected by saturation binding. Second, inhibition plots from bicuculline and bicuculline methiodide inhibition experiments in bullfrog brain suggested the presence of two classes of binding sites [14]. In the mammalian brain, the low affinity binding site of the GABA<sub>A</sub> receptor preferentially binds [ $^3\text{H}$ ]bicuculline methochloride [49]. Thus, the use of a different radiolabeled analog (such as [ $^3\text{H}$ ]bicuculline, or [ $^3\text{H}$ ]bicuculline methochloride) might identify more GABA<sub>A</sub> receptor subtypes in the bullfrog brain.

The density of [ $^3\text{H}$ ]muscimol binding sites ( $B_{\text{max}} = 1.8$  pmol/mg protein) in bullfrog brain membranes was similar to bovine cerebral cortex (3.5 pmol/mg protein), and codfish brain (2.73 pmol/mg protein) [2,19]. Furthermore, the number of [ $^3\text{H}$ ]muscimol binding sites was similar to the number of binding sites observed with [ $^3\text{H}$ ]GABA (1.6 pmol/mg protein) in the same species [26]. [ $^3\text{H}$ ]GABA can theoretically bind to all three classes of GABA receptors while [ $^3\text{H}$ ]muscimol should bind only to the GABA<sub>A</sub> class. The similarity in the number of sites detected with these two ligands suggests that the GABA<sub>A</sub> class constitutes the vast majority of GABA receptors in bullfrog brain. The concentration of [ $^3\text{H}$ ]muscimol binding sites in *R. esculenta* was also estimated with in vitro quantitative autoradiography [54]. The range of concentrations across brain areas was within an order of magnitude of our estimate from bullfrog whole brain homogenates. In contrast, there are markedly lower numbers of [ $^3\text{H}$ ]GABA binding sites observed in brain membranes of salmon (16.6–41.4 fmol/mg protein) [27].

The GABA<sub>A</sub> receptor agonists and antagonists inhibited [ $^3\text{H}$ ]muscimol specific binding in a concentration-dependent manner. The rank order of potency was muscimol>GABA>bicuculline methiodide>bicuculline. GABA is also a more potent inhibitor than bicuculline when [ $^3\text{H}$ ]GABA is the labeled ligand [26]. This rank order is similar to that seen in rat, bovine, and codfish brain membranes, with muscimol being the most potent competitor followed by GABA and bicuculline methiodide and/or bicuculline [2,19,51]. The  $\text{EC}_{50}$  of bicuculline methiodide in bullfrog brain (13.2  $\mu\text{M}$ ) was very similar to that of the codfish (15.6  $\mu\text{M}$ ) [19]. However, both muscimol and GABA had higher  $\text{EC}_{50}$ s than observed in other vertebrates. Finally, in bullfrogs, the mammalian GABA<sub>B</sub> receptor agonist baclofen did not inhibit [ $^3\text{H}$ ]muscimol binding. This supports the hypothesis that [ $^3\text{H}$ ]muscimol binding in bullfrog brain is to a GABA<sub>A</sub>-like receptor, rather than a GABA<sub>B</sub>-like receptor [39]. The pharmacological properties of the GABA<sub>A</sub>-like receptor in



the bullfrog brain are thus similar to receptors in other vertebrates. Species differences in  $EC_{50}$ s may indicate that the bullfrog GABA<sub>A</sub> receptor differs structurally.

In summary, the bullfrog brain possesses a single class of high affinity binding sites for the GABA<sub>A</sub> analog, [<sup>3</sup>H]muscimol. This is the first thorough characterization of the binding of any GABA analog in the amphibian brain. The binding kinetics, saturation binding characteristics, and ligand specificity for this analog support the hypothesis that the brain of this species contains a GABA<sub>A</sub>-like receptor. The characteristics of this receptor are substantially similar to the characteristics of the GABA<sub>A</sub> receptor in other vertebrates.

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