

Acute neurosteroid modulation and subunit isolation of the γ -aminobutyric acid_A receptor in the bullfrog, *Rana catesbeiana*

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Abstract

The inhibitory neurotransmitter γ -aminobutyric acid (GABA) has multiple receptors. In mammals, the GABA_A receptor subtype is modulated by neurosteroids. However, whether steroid interaction with the GABA_A receptor is unique to mammals or a conserved feature in vertebrates is unknown. Thus, neurosteroid modulation of the GABA_A receptor was investigated in the brain of the bullfrog (*Rana catesbeiana*) using the mammalian GABA_A receptor agonist [³H]muscimol. Two neurosteroids, allopregnanolone and pregnenolone sulfate, affected [³H]muscimol specific binding in bullfrog brain membrane preparations. Allopregnanolone significantly increased [³H]muscimol specific binding in a dose- and time-dependent manner. The pattern of allopregnanolone modulation supports the hypothesis that the bullfrog brain possesses both high-affinity and low-affinity [³H]muscimol binding sites. Unlike allopregnanolone, pregnenolone sulfate showed biphasic modulation with increased [³H]muscimol specific binding at low nanomolar concentrations and decreased specific binding at micromolar concentrations. Additionally, three cDNA fragments with significant homology to mammalian GABA_A receptor subunits were isolated from the bullfrog brain. These fragments belong to the α 1, β 1, and γ 2 subunit families. In mammals, GABA_A receptors composed of these specific subunit isoforms are effectively modulated by neurosteroids, including allopregnanolone. Neurosteroid modulation of the amphibian brain GABA_A receptor is therefore supported by both [³H]muscimol binding studies and subunit sequences. Allopregnanolone and pregnenolone sulfate modulation of this receptor may thus represent a significant mechanism for steroid influence on amphibian brain and behavior.

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Introduction

The vast majority of fast inhibitory synaptic transmission in the vertebrate central nervous system occurs through the binding of the amino acid neurotransmitter, γ -aminobutyric acid (GABA), to the GABA_A receptor. In mammals, radioligand binding studies indicate that the GABA_A receptor has many different subtypes (Knapp *et al.* 1990). The binding properties of these receptor subtypes are based on different combinations of six classes of subunit proteins (α 1–6, β 1–3, γ 1–3, δ , ϵ , and π) (Watanabe *et al.* 2002).

Similarly, subunit composition also determines if and how neurosteroids modulate receptor function (Paul & Purdy 1992, Hauser *et al.* 1997, Akk *et al.* 2001, Findlay *et al.* 2000). Molecular cloning of the subunits, coupled with photo-affinity labeling and point mutation studies, has led to the identification of specific binding domains and structural characteristics of receptor subtypes (Smith & Olsen 1995). Despite significant advances in understanding the mammalian receptor (Tyndale *et al.* 1995, Baulieu 1997, 1998, Compagnone & Mellon 2000, Watanabe *et al.* 2002), relatively fewer studies have examined the

GABA_A receptor in non-mammals (Anzelius *et al.* 1995, Aller *et al.* 2003).

There is functional evidence for the presence of a GABA_A receptor in amphibians. GABA itself is broadly distributed across the amphibian brain (Franzoni & Morino 1989, Antal 1991, Hamilton 1992, Kratskin *et al.* 1992, Reichenberger *et al.* 1993, Naujoks-Manteuffel *et al.* 1994, Rybicka & Udin 1994, Barale *et al.* 1996, Li & Fite 1998, Pollák *et al.* 1999, Endepols *et al.* 2000, Jimenez *et al.* 2000). GABA, via the GABA_A receptor, alters α -melanocyte-stimulating hormone release in the neurointermediate lobe and inhibits the biosynthesis of neurosteroids in the hypothalamus of the frog *Rana ridibunda* (Adjeroud *et al.* 1986, Do-Régo *et al.* 1998, Mensah-Nyagan *et al.* 2001a). GABA_A receptor ligands influence both olfactory and auditory integration in frogs (Duchamp-Viret *et al.* 1993, Zhang *et al.* 1999, Zheng & Hall 2000, Lin & Feng 2003). An unusual mechanism for GABA action may exist in the frog optic tectum (Nistri & Sivilotti 1985, Sivilotti & Nistri 1989, 1992, Mazda *et al.* 1990), where GABA is involved in visual processing (Li & Fite 2001). Finally, a GABA_A receptor-mediated response to stress has been implicated in newt behavior (Boyd & Moore 1990).

The presence of a GABA_A-like receptor in the amphibian central nervous system is supported by membrane-binding and autoradiography studies with the analogs [³H]muscimol, [³H]flunitrazepam, [³H]Ro 15-1788, and [³⁵S]t-butylbicyclophosphorothionate (Enna & Snyder 1977, Schmitz *et al.* 1988, Yang *et al.* 1992, Tavolaro *et al.* 1993, Orchinik *et al.* 1994, Hollis & Boyd 2003). In addition, antibodies to mammal β 2/ β 3 subunits recognize a β -like form in the amphibian brain and pituitary (Aller *et al.* 1997, Louiset *et al.* 2000). No GABA receptor proteins have been isolated or cloned in any amphibian.

In mammals, the neurosteroid allopregnanolone is a potent positive modulator of the GABA_A receptor (for review see Lambert *et al.* 1995), while pregnenolone sulfate acts as an antagonistic inhibitor (Majewska & Schwartz 1987). There is strong evidence for allopregnanolone and pregnenolone sulfate binding sites on the newt (*Taricha granulosa*) and ranid (*R. ridibunda*) GABA_A receptor respectively (Orchinik *et al.* 1994, Le Foll *et al.* 1997). The enzymes P450 scc, which converts cholesterol to pregnenolone, and hydroxysteroid

sulfotransferase, which converts pregnenolone to pregnenolone sulfate, have both been found in frog brain (Beaujean *et al.* 1999, Takase *et al.* 1999, Mensah-Nyagan *et al.* 2000, 2001a,b). Pregnenolone sulfate itself has been identified in frog brain using RIA (Takase *et al.* 1999). The enzyme 3β -hydroxysteroid dehydrogenase which converts pregnenolone to progesterone, has been localized in the amphibian brain (*R. ridibunda*) by immunocytochemistry (Mensah-Nyagan *et al.* 1994, Do-Régo *et al.* 1998). Allopregnanolone, a progesterone metabolite, is produced in the brain of bullfrog tadpoles when injected with progesterone (Mok & Kreiger 1990, 1992). It is thus likely that the amphibian brain can produce allopregnanolone and pregnenolone sulfate. The function of these neurosteroids in the frog brain is unknown.

In this study, neurosteroid modulation of the GABA_A receptor was investigated in the brain of the bullfrog (*Rana catesbeiana*) through binding assays using the GABA_A receptor agonist [³H]muscimol. In mammals, GABA_A receptors composed of specific subunit isoforms are modulated by neurosteroids, particularly by allopregnanolone (Lan *et al.* 1990, Puia *et al.* 1990, Pistis *et al.* 1997, Lambert *et al.* 1999). Based on the subunit composition of the mammalian GABA_A receptor (Pritchett *et al.* 1989b), molecular cloning was used to determine whether bullfrog brain contained homologous genes for the α , β , and γ subunits. The presence of specific subunit isoforms would support the hypothesis that neurosteroids modulate the bullfrog GABA_A receptor.

Materials and methods

Animals

Adult male bullfrogs were purchased from C. Sullivan Company (Nashville, TN, USA). Animals were housed in the laboratory on a 12 h light:12 h darkness controlled photoperiod at 17 °C in large tanks (50 × 21 × 21 cm) with flow-through water. They were maintained on a diet of goldfish. Bullfrogs were cryoanesthetized, rapidly decapitated, and the brains removed 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 Institutional Animal Care and Use Committee (IACUC).

Membrane binding assays

Preparation of bullfrog brain membranes and the termination of assays with vacuum filtration has been previously described (Hollis & Boyd 2003). Unless otherwise noted, all chemicals and incubations were kept at 4 °C or on ice. Under these conditions, the binding of 20 nM [³H]muscimol to bullfrog membranes reaches equilibrium in less than 3 min. The design of neurosteroid modulation experiments was based first on previously published results from rats and then systematically modified based on results in bullfrogs. All binding data were analyzed using GRAPHPAD PRISM (v. 3.0; Graph Pad, San Diego, CA, USA). This software performed transformations, non-linear regression curve fitting, F-tests, and ANOVA. Values of $P < 0.05$ were considered significant.

Allopregnanolone modulation experiments followed the methods of Mehta & Ticku (1999), with modifications. Allopregnanolone (5 α -pregnan-3 α -ol-20-one; Steraloids, Newport, RI, USA) was initially solubilized in 40% 2-hydroxypropyl- β -cyclodextrin (45% w/v; Research Biochemical International, Natick, MA, USA) in buffer at a concentration of 10 mM before being made into stock solutions. Increasing concentrations of allopregnanolone were added to membrane receptor preparations preincubated at various times with 20 nM [³H]muscimol. After allopregnanolone was added, the membrane receptor preparations were incubated for an additional 30 min. After 30 min of [³H]muscimol/allopregnanolone incubation, the reactions were terminated. In control experiments where allopregnanolone was solubilized in ethanol, rather than cyclodextrin, increases in specific binding averaged 50%. The EC₅₀ values fell within the 10⁻⁷ and 10⁻⁶ M range. Cyclodextrin alone did not affect [³H]muscimol binding to bullfrog brain membranes.

Pregnenolone sulfate modulation experiments followed the methods of Majewska *et al.* (1985), with modifications. Pregnenolone sulfate (5 pregnen-3 β -ol-20-one sulfate; Steraloids) was initially solubilized in buffer at a concentration of 1 mM. Aliquots of 20 nM [³H]muscimol were added to

membrane receptor preparations, preincubated for different lengths of time, in increasing concentrations of pregnenolone sulfate. After [³H]muscimol was added, the membrane receptor preparations were incubated for an additional 20 min. After 20 min of [³H]muscimol and pregnenolone sulfate incubation, the reactions were terminated.

Cloning of $\alpha 1$, $\beta 1$, and $\gamma 2$ subunit isoforms

Cloning followed the methods of Roberts *et al.* (2000), with modifications. Following decapitation, frog brains were immediately homogenized (Polytron, Brinkman Instruments, Inc., Westbury, NY, USA) in Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) at a ratio of 50 mg tissue/1.0 ml reagent to extract total RNA (Chomczynski & Sacchi 1987, Chomczynski 1993). The RNA obtained was dissolved in FORMAZOL (Molecular Research Center, Inc.) and held at -20 °C.

Two sets of degenerate primers for each subunit were designed based on the alignments of published GABA_A receptor subunit isoform sequences. The $\alpha 1$ and $\gamma 2$ degenerate primers were designed against mammalian and chicken GABA_A $\alpha 1$ and $\gamma 2$ sequences, as well as the zebrafish GABA_A $\gamma 2$ sequence for the $\gamma 2$ primers. The GABA_A $\beta 1$ degenerate primers were originally designed from the alignments of mammalian $\beta 2$ and invertebrate (cuttlefish, snail, and *Drosophila*) β subunits with the intention of isolating the $\beta 2$ subunit. The primers were $\alpha 1$ (forward, 5'-GGG AGA GCG TGT AAC YGA AGT GAA GAC BGA-3'; reverse, 5'-GCT GAC RCT GTT AAA RGT TTT CTT GGG TTC-3'), $\beta 1$ (forward, 5'-CTA AAG GGA TGY KGA GAG TSC GGA AAA RGG G-3'; reverse, 5'-CSC CRC GCC AGT AAA AYT CAA TGT CRT CWG T-3'), and $\gamma 2$ (forward, 5'-CCA ATG GAT GMV CAY TCS TGY CCY CTS GA-3'; reverse, 5'-AAG CAV ACW GAY ACR AAR AGR TCC ATH GCY-3').

Messenger RNA from bullfrog brain total RNA was reverse transcribed using AMV reverse transcriptase (cDNA cycle; Invitrogen, San Diego, CA, USA) and the resulting cDNA was used for PCR (Advantage KlenTaq Polymerase Mix; Clontech, Palo Alto, CA, USA). Each reaction (94 °C, 2 min; 56 °C ($\alpha 1$, $\gamma 2$) or 51 °C ($\beta 1$), 2 min;

72 °C, 90 sec; 30 cycles ($\alpha 1$, $\gamma 2$) or 40 cycles ($\beta 1$); (PTC100; MJ Research, Waltham, MA, USA) for each subunit isoforms' set of primers was run separately. PCR reactions were separated on agarose gels, visualized under u.v. light and the appropriate sized bands were gel purified. Products were ligated into TOPO vector pCR 2.1 (Invitrogen, Carlsbad, USA) and transformed in *E. coli*. Positive colonies were grown for plasmid preparations and the cDNA sequenced using simultaneous bidirectional sequencing reactions with the DYEnamic cycling sequencing kit (US79535) from Amersham Pharmacia Biotech (Baie d'Urde, Quebec, Canada) utilizing internal primers (LiCor, Lincoln, NB, USA) for the plasmid BK and T7 promoter regions. The sequencing reactions were separated and analyzed using an ALFexpress Sequencer (Pharmacia Biotech, Piscataway, NJ, USA). Sequence analysis and alignments were performed using MacVector 6.5 software (Oxford Molecular Ltd, Genetics Computer Group, Madison, WI, USA).

First-strand cDNA generated using the degenerate GABA_A $\alpha 1$, $\beta 1$, and $\gamma 2$ primers produced fragments of 1069, 633, and 436 base pairs. Of these, 1063, 630, and 377 base pairs were successfully sequenced respectively. Hydrophobicity plots of each isolated fragment (generated with the MacVector Janin hydrophobicity method; Miller *et al.* 1987) predicted regions considered transmembrane spanning domains. Comparisons using the Basic Local Alignment Search Tool (NCBI, Bethesda, MD, USA; www.ncbi.nlm.nih.gov/BLAST/) to sequences in GenBank determined similarity of each cDNA fragment to known GABA_A receptor sequences. Each cDNA fragment was most homologous with the mammalian subunit isoform from which it was designed, except the $\beta 2$ sequence, which showed greater homology to the $\beta 1$ isoform.

Results

Membrane binding assays

Allopregnanolone caused both dose- and time-dependent positive modulation of [³H]muscimol specific binding in bullfrog brain membrane preparations (Fig. 1). Higher concentrations of allopregnanolone, in combination with increased [³H]muscimol preincubation times, resulted in

increased radioactive ligand binding. [³H]muscimol preincubation for 10 min, 20 min and 30 min, before the addition of allopregnanolone, caused specific binding to plateau at levels 30%, 42% and 76% greater than the controls respectively. Preincubation with [³H]muscimol for either 20 min or 30 min before the addition of allopregnanolone caused significant overall increases in [³H]muscimol specific binding compared with the control ($P < 0.0001$; ANOVA). Preincubation with [³H]muscimol for 10 min before the addition of allopregnanolone also caused a significant increase; however, this was not as pronounced ($P < 0.05$; ANOVA). When [³H]muscimol and allopregnanolone were incubated together, without preincubation, allopregnanolone had no effect on [³H]muscimol binding.

Comparisons between preincubation times showed that [³H]muscimol preincubation times of 30 min, before the addition of allopregnanolone, caused significantly greater increases in [³H]muscimol specific binding than shorter preincubation periods (F-test comparison of non-linear regression curves with data normalized to % increase of control; 30 min vs 20 min, $P < 0.05$; 30 min vs 10 min, $P < 0.001$). Although preincubation times of 20 min showed significantly decreased [³H]muscimol specific binding compared with 30 min ($P < 0.05$; F-test), it was significantly greater than the 10-min preincubation times ($P < 0.001$; F-test).

In addition to the influence of allopregnanolone on [³H]muscimol specific binding, there was also a noticeable shift regarding the EC₅₀ values at the different [³H]muscimol preincubation times. There was a systematic decrease in EC₅₀ values with decreased [³H]muscimol preincubation times (30 min = 1.308 μ M, 20 min = 0.339 μ M, 10 min = 0.149 μ M). The greatest EC₅₀ shift occurred between the [³H]muscimol preincubation times of 20 and 30 min (0.961 μ M). The EC₅₀ shift between 10- and 20-min preincubation times (0.190 μ M) was only about 20% of the EC₅₀ shift between 20 and 30 min.

Pregnenolone sulfate also modulated [³H]muscimol specific binding in bullfrog brain membrane preparations (Fig. 2). Preincubation with pregnenolone sulfate for 30 min, before the addition of [³H]muscimol, was associated with a significant increase in [³H]muscimol specific binding at the lower nanomolar concentrations. This peak of 111% above control was reached at 1 nM

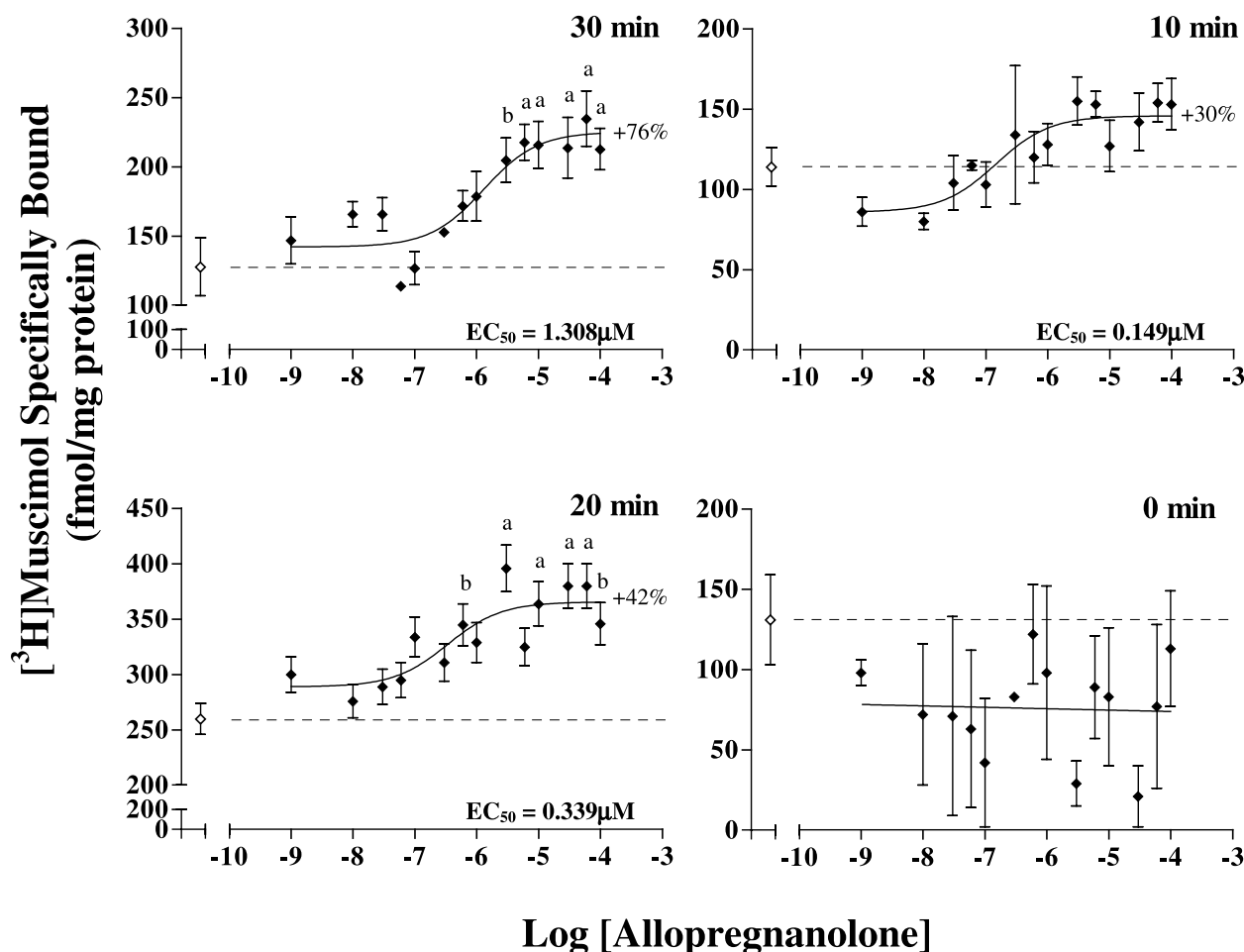


Figure 1 Concentration- and time-dependent positive modulation of [³H]muscimol binding by allopregnanolone in bullfrog brain membranes. Times in the upper right corner of each panel indicate the period of [³H]muscimol preincubation. The addition of increasing concentrations of allopregnanolone, in combination with increased [³H]muscimol preincubation times, resulted in increases of specifically bound [³H]muscimol (30 and 20 min, $P < 0.0001$; 10 min, $P < 0.05$; 0 min, not significant; ANOVA). Control (\diamond and broken lines). Percent increase from control at plateau is indicated. Data are the means \pm S.E.M. of triplicate determinations from representative experiments. ^a $P < 0.001$ and ^b $P < 0.05$; Dunnet's *post hoc* test.

($P < 0.05$; ANOVA, Dunnet's *post hoc*). Pregnenolone sulfate did not significantly affect [³H]muscimol binding at preincubation times of 10 min. Comparisons between pregnenolone sulfate preincubation times showed that the alteration in [³H]muscimol specific binding with 30-min preincubation was significantly different from the 10-min preincubation period ($P < 0.0005$; F-test comparison of non-linear regression curves with data normalized to % increase of control). Preincubation times of 20 min (data not shown) did not significantly affect binding either.

Cloning of the bullfrog GABA_A receptor $\alpha 1$, $\beta 1$, and $\gamma 2$ subunit

Three isolated cDNA fragments indicated GABA_A receptor $\alpha 1$ -like (accession no. AF411006; Fig. 3), $\beta 1$ -like (accession no. AF411007; Fig. 4), and a $\gamma 2$ -like (accession no. AF394748; Fig. 5) subunit isoforms in the bullfrog brain. The $\alpha 1$ -like fragment sequence was 1063 bp (354 amino acids), while the $\beta 1$ -like subunit partial sequence was 630 bp (210 amino acids), and the $\gamma 2$ partial sequence was 377 bp (126 amino acids). The discrepancy

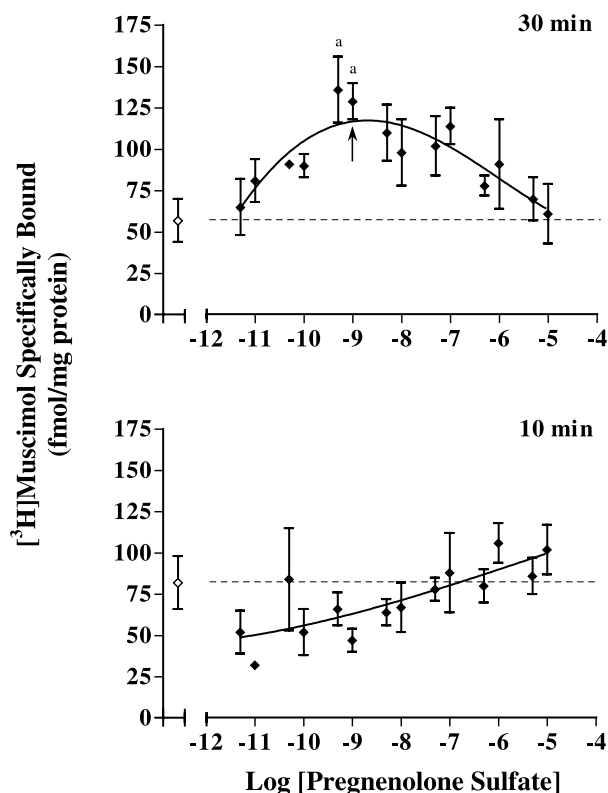


Figure 2 Concentration- and time-dependent positive modulation of [³H]muscimol binding by pregnenolone sulfate in bullfrog brain membranes. The addition of increased concentrations of pregnenolone sulfate, in combination with a minimum preincubation time of 30 min (top), resulted in a significant, bimodal change in [³H]muscimol bound ($P < 0.05$; ANOVA). Preincubation time of 10 min (bottom) resulted in no significant change. Control (\diamond and broken lines). The arrow indicates the peak percent increase at plateau. Data are the means \pm S.E.M. of triplicate determinations from representative experiments. ^a $P < 0.05$; Dunnett's post hoc test.

between nucleotide number and amino acid number for the $\alpha 1$ -like subunit was due to a single nucleotide (G) at the 5' end, while the $\gamma 2$ -like subunit discrepancy was due to the final two nucleotides (A and C) invariably coding for threonine, regardless of the non-sequenced, third nucleotide.

Bullfrog GABA_A receptor amino acid partial sequences showed high homology with those of other vertebrates. The bullfrog $\alpha 1$ -like subunit had greatest percentage of shared sequence identity with the GABA_A receptor $\alpha 1$ subunits of the chicken (94.6%) and the rat (93.7%). The $\beta 1$ -like

subunit partial sequence of the bullfrog aligned with the highest sequence identity to the GABA_A receptor $\beta 1$ subunit of mammals, including the rat (93.8%), which was merely 2.4% higher than seen with the $\beta 2$ subunits of the rat (91.4%) and zebrafish ($\beta Z 2$) (91.4%). The bullfrog $\beta 1$ -like subunit partial sequence showed higher sequence identity with the β subunits of invertebrates than it did the vertebrate α and γ isoforms. The greatest amount of homology was seen within the transmembrane domains. The $\gamma 2$ -like subunit partial sequence of the bullfrog aligned with the highest sequence identity to the GABA_A receptor $\gamma 2$ subunit of mammals, including the rat (94.4%).

Based on multiple alignments with GABA_A receptor subunits from the rat, the $\alpha 1$ -like subunit partial sequence of the bullfrog was entirely within the open reading frame and represented 77.8% of the rat $\alpha 1$ subunit (82.7% of the mature $\alpha 1$ sequence; Tyndale *et al.* 1995). Over one-half of the entire fragment represented part of the N-terminal extracellular domain, while the remaining 47% spanned three of the putative four transmembrane domains and part of the intracellular loop. In addition, the bullfrog $\alpha 1$ -like subunit contained multiple conserved regions and residues associated with agonist photoaffinity labeling, binding sites, and channel gating sites (see Discussion).

The bullfrog $\beta 1$ -like and $\gamma 2$ -like subunit fragments represented approximately 44.3% of the rat $\beta 1$ sequence (46.8% of the mature sequence; Tyndale *et al.* 1995) and 27.0% of the rat $\gamma 2$ sequence (29.4% of the mature sequence; Tyndale *et al.* 1995) respectively. Both fragments were entirely within the open reading frames and spanned part of the N-terminal extracellular region. The $\beta 1$ -like subunit was sequenced midway through the M2 transmembrane domain, while the $\gamma 2$ -like sequence extended just past the M2 transmembrane domain. In addition, the bullfrog $\beta 1$ -like and $\gamma 2$ -like subunit fragments contained multiple conserved regions and residues (see Figs 4 and 5 and Discussion).

Those regions of the bullfrog partial sequences that corresponded with transmembrane domains of other vertebrates showed the highest homology (Figs 3, 4 and 5.) The bullfrog $\alpha 1$ -like subunit partial sequence spanned three regions that corresponded (100% identity) with the rat and chicken GABA_A receptor $\alpha 1$ subunit M1, M2 and M3 transmembrane domains. The bullfrog $\beta 1$ -like

| | | |
|--|---|------------------|
| <i>Rana catesbeiana</i> (α 1-like) | | 5' . . . |
| <i>Gallus gallus</i> (α 1) | | 5' . . . 61 |
| <i>Rattus norvegicus</i> (α 1) | | 5' . . . 60 (33) |
| | | |
| <i>Rana catesbeiana</i> (α 1-like) | GERVTEVKTDIFVTSFGPVS DHDMEYTTIDVFFRQGWDRERLKFKGPMTVLRNLNLMASKIWTPTDFF | |
| <i>Gallus gallus</i> (α 1) | -----S-K----- | 128 |
| <i>Rattus norvegicus</i> (α 1) | -----S-K----- | 127 (100) |
| | | |
| <i>Rana catesbeiana</i> (α 1-like) | HNGKKSVAHNMTMPNKLRLRITEDGTLTYMLRTVRAECPMHLEDFPMDAHACPLKFGSYAYTRAEVV | |
| <i>Gallus gallus</i> (α 1) | -----V----- | 195 |
| <i>Rattus norvegicus</i> (α 1) | ----- | 194 (167) |
| | | |
| <i>Rana catesbeiana</i> (α 1-like) | YTWTREPAQSVVVAEDGSRNLNQYDLLGLNVASGIVQSSTGEYVVMTHFHLKRKIGYFVIQTYLPCI | |
| <i>Gallus gallus</i> (α 1) | -E-----R-----QT-D----- | 262 |
| <i>Rattus norvegicus</i> (α 1) | -E-----R-----QT-D----- | 261 (234) |
| | | |
| <i>Rana catesbeiana</i> (α 1-like) | MTVILSQVSPFWLNRESVPARTVFGVTTVLTMTTLLSISARNSLPKVAYATAMDWFIACVYAFVFSALI | |
| <i>Gallus gallus</i> (α 1) | ----- | 329 |
| <i>Rattus norvegicus</i> (α 1) | ----- | 328 (301) |
| | | |
| <i>Rana catesbeiana</i> (α 1-like) | EFATVNYFTKQGYAWDGKSVVPEKPKVKDPLIKKNNTYASATANYPP* IAR*DPGLATTIAKSATIE | |
| <i>Gallus gallus</i> (α 1) | -----R-----TA-ATS-T-N-*----- | 395 |
| <i>Rattus norvegicus</i> (α 1) | -----R-----PTATS-T-NL--G----- | 395 (368) |
| | | |
| <i>Rana catesbeiana</i> (α 1-like) | PKEIEPKPKPAEPKKTFSVS . . . 3' | |
| <i>Gallus gallus</i> (α 1) | ---VK-ET----- . . . 3' 416 [94.6%] | |
| <i>Rattus norvegicus</i> (α 1) | ---VK-ET--P----- . . . 3' 416 (389) [93.7%] | |

Figure 3 Alignment of the bullfrog α 1-like subunit cDNA fragment with fragments encoding amino acid sequences of α 1 subunits from different vertebrate species. Dashes indicate amino acid residues identical to those of *Rana catesbeiana*. Asterisks indicate gaps, while bold lines indicate putative transmembrane domains. To the right, amino acid numbers are shown and the mature α 1 subunit amino acid number of *Rattus norvegicus* (Tyndale *et al.* 1995) is in parentheses. The total number of identical amino acid residues (out of 354 possible) with the α 1-like fragment of *Rana catesbeiana* for each species is indicated at the end of each sequence with percent identity in parentheses. Sequences were acquired from GenBank. Accession numbers: *Rana catesbeiana*, AF411006; *Gallus gallus*, P19150; *Rattus norvegicus*, AAC42029.

subunit partial sequence spanned a region that corresponded (100% identity) with the rat β 1 subunit M1 transmembrane domain and extended 16 amino acids into the M2 transmembrane domain. Finally, the bullfrog γ 2-like subunit also showed 100% identity with the rat, chicken, and white perch γ 2 subunit M1 and M2 transmembrane domains.

Discussion

The constituent subunits necessary to form a functional GABA_A receptor in the bullfrog brain were identified here for the first time in any amphibian. Three cDNA fragments were isolated and found to be highly homologous to GABA_A receptor subunit isoforms of other vertebrates. Thus, these fragments indicated the presence of

GABA_A receptor α 1-like, β 1-like, and γ 2-like subunits in the bullfrog brain. The subunits that make up a mammalian GABA_A receptor form a pentameric structure (Nayeem *et al.* 1994). A functional GABA_A receptor is most likely to be assembled from a combination of α , β , and γ subunits (Pritchett *et al.* 1989*a,b*, Malherbe *et al.* 1990*a,b*, Olsen & Tobin 1990, Weiland *et al.* 1992). Different combinations of these subunits result in the endogenous heterogeneity of the GABA_A receptor (Burt & Kamatchi 1991, Olsen *et al.* 1991). Whether the subunits identified in the bullfrog are assembled together is not known. Within the bullfrog GABA_A receptor α 1-like subunit, two very highly conserved tryptophan residues (Trp-69 and Trp-94 of the rat α 1 subunit) were present. In mammals, these residues are critical in the formation of the interface between α and β subunits (Srinivasan *et al.* 1999). Thus, the

| | | | |
|--|---|--------|-------------------|
| <i>Rana catesbeiana</i> (γ 2-like) | | 5' ... | |
| <i>Rattus norvegicus</i> (γ 2) | | 5' ... | 206 (168) |
| <i>Gallus gallus</i> (γ 2) | | 5' ... | 206 |
| <i>Morone americana</i> (γ 2) | | 5' ... | 203 |
| | | | |
| <i>Rana catesbeiana</i> (γ 2-like) | FSSYGYPKEEIIYQWKRSSVEVDIRSWRLYQFSFVGLRNTTEEVKTTSGDYVVMTVYFDLARRMGY | | |
| <i>Rattus norvegicus</i> (γ 2) | -----R---V-----T-----V-----S---S----- | | 273 (235) |
| <i>Gallus gallus</i> (γ 2) | -----R-----T-----T-----V-----S---N-S----- | | 273 |
| <i>Morone americana</i> (γ 2) | -----R---V-K-----S-I-R-V-----L-F---S----- | | 270 |
| | | | |
| <i>Rana catesbeiana</i> (γ 2-like) | FTIQTYIPCTLIIVVLSWVSWFWINKDAVPARTSLGITTTLTMTTLSTIRRKSLPKVSYV | | |
| <i>Rattus norvegicus</i> (γ 2) | -----A----- | ... 3' | 331 (293) [94.4%] |
| <i>Gallus gallus</i> (γ 2) | -----A----- | ... 3' | 331 [93.7%] |
| <i>Morone americana</i> (γ 2) | -----A----- | ... 3' | 328 [91.3%] |

Figure 5 Alignment of the bullfrog γ 2-like subunit cDNA fragment with fragments encoding amino acid sequences of γ 2 subunits of different vertebrate species. Dashes indicate amino acid residues identical to those of *Rana catesbeiana*. Bold lines indicate putative transmembrane domains. To the right, amino acid numbers are shown and the mature γ 2 subunit amino acid number of *Rattus norvegicus* (Tyndale *et al.* 1995) is in parentheses. The total number of identical amino acid residues (out of 125 possible) with the γ 2-like fragment of *Rana catesbeiana* for each species is indicated at the end of each sequence with percent identity in parentheses. Sequences were acquired from GenBank. Accession numbers: *Rana catesbeiana*, AF394748; *Rattus norvegicus*, AAC42036; *Gallus gallus*, CAA38704; *Morone americana*, AAL50804.

conserved tryptophan residues may perform a similar function in α and β subunit association for the formation of the pentameric GABA_A receptor structure in the bullfrog brain.

The neurosteroid allopregnanolone modulated the binding of the GABA_A receptor agonist [³H]muscimol in bullfrog brain membranes. This modulation is consistent with the subunits identified. In mammals, sensitivity of the GABA_A receptor to modulation by neurosteroids depends on subunit combinations (for review see Compagnone & Mellon 2000). For example, the combination of mammalian α 1 β 1 γ 2 forms a GABA_A receptor which is modulated by allopregnanolone (Lan *et al.* 1990, Pistis *et al.* 1997, Lambert *et al.* 1999). Thus, the subunit combination of α 1, β 1, and γ 2 in the bullfrog supports the hypothesis that allopregnanolone modulation of the GABA_A receptor is conserved in the amphibian brain. In mammals, the GABA-potentiating effect of allopregnanolone is found to be greatest for α 1 (over α 2 and α 3) subunit containing receptors (Shingai *et al.* 1991). Modulatory effects of allopregnanolone are preserved in cells transfected with α 1 β 1 γ 2L, α 1 β 1 and β 1 only subunits (Puia *et al.* 1990). This suggests that a neurosteroid binding site is likely located on the β 1 subunit and that the modulatory effects of allopregnanolone are maintained when combined with α 1 and γ 2L subunits. Substitution of the serine at position 265 on the β 1 subunit dramatically decreases the enhancement of GABA-induced

currents by allopregnanolone, which also supports the hypothesis that the allopregnanolone binding site is on β 1 (Findlay *et al.* 2000). Bullfrogs possess this critical serine at the equivalent position on the β 1 subunit.

Allopregnanolone caused a dose-dependent positive modulation of [³H]muscimol specific binding in bullfrog brain membrane preparations. This positive modulation is also seen in the rat brain (Goodnough & Hawkinson 1995, Hauser *et al.* 1995, Mehta & Ticku 1999). However, a striking difference was seen in timing in rats and bullfrogs. In the rat, an enhancement of [³H]muscimol binding is seen with the simultaneous addition of allopregnanolone and [³H]muscimol (Goodnough & Hawkinson 1995, Mehta & Ticku 1999), or preincubation with allopregnanolone (Lopez-Colome *et al.* 1990). No indication of [³H]muscimol binding enhancement was observed in the bullfrog membrane preparations when allopregnanolone was added at the same time as [³H]muscimol. Positive modulation in bullfrog membranes by allopregnanolone occurred only after 10 min or longer preincubation time with [³H]muscimol. Thirty minutes of [³H]muscimol preincubation allowed the greatest allopregnanolone enhancement of [³H]muscimol specific binding. The mean percent increase to 76% above control was similar to the enhancement seen in mammals (Peters *et al.* 1988, Lopez-Colome *et al.* 1990, Hauser *et al.* 1995, Mehta & Ticku 1999). The differences seen

between the bullfrog and mammals may reflect differences in GABA_A receptor subtype structure, levels of subtype expression, membrane composition or assay temperature.

The modulation of both a high- and low-affinity binding site by allopregnanolone is the first indication of a low-affinity [³H]muscimol binding site in an amphibian brain. Interestingly, increases in [³H]muscimol preincubation times shifted the EC₅₀ of allopregnanolone toward larger values. Allopregnanolone EC₅₀ values observed with [³H]muscimol binding in rats show a high-affinity site with EC₅₀ of 64 nM and a low-affinity site with EC₅₀ of 1.8 μM (Goodnough & Hawkinson 1995). The gradual shift in EC₅₀ seen in bullfrogs may have reflected a shift from [³H]muscimol high-affinity binding enhancement to increases in low-affinity [³H]muscimol binding enhancement. Binding of [³H]GABA itself to brain and spinal cord membranes of the bullfrog does not indicate the presence of a low-affinity site (Enna & Snyder 1977). The presence of a low-affinity site in bullfrogs is thus supported for the first time by this allopregnanolone modulation study.

Pregnenolone sulfate also modulated the binding of [³H]muscimol to bullfrog brain membranes. Little is known about GABA receptor subunit specificity of pregnenolone sulfate. However, similar to picrotoxin, it antagonizes the GABA mediated Cl⁻ current, but at a different binding site (Majewska & Schwartz 1987, Akwa *et al.* 1991, Shen *et al.* 1999). Mutation to the γ2 subunit, eliminating picrotoxin sensitivity, does not block the antagonistic properties of pregnenolone sulfate (Shen *et al.* 1999). However, mutation to the α1 subunit residue, Val-256, found in the bullfrog α1-like subunit as well (arbitrary position 223), creates a conformational change that underlies the blocking effect of pregnenolone sulfate (Akk *et al.* 2001). Homology between this residue in the bullfrog and mammals suggests conservation in the effects of pregnenolone sulfate in bullfrogs as well.

Pregnenolone sulfate showed a distinct biphasic effect on [³H]muscimol binding only when membranes were preincubated with the steroid for 30 min. No modulation was observed with shorter preincubation times. This result is relatively consistent with the equilibrium binding of [³H]pregnenolone sulfate, which is about 20 min (Majewska *et al.* 1990). The lack of an effect at

20 min may be due to the assay being performed at 4 °C, to keep [³H]muscimol binding experiments consistent. The optimum specific binding temperature for [³H]pregnenolone sulfate in rat synaptosomal membranes is 25 °C (Majewska *et al.* 1990). Pregnenolone sulfate modulation in the bullfrog was otherwise similar to modulation in mammals (Majewska *et al.* 1985, Hauser *et al.* 1995). Binding of [³H]muscimol in the bullfrog brain, however, significantly increased between 100 pM and 1 nM concentrations of pregnenolone sulfate, as opposed to 10 nM, as seen in mammals. The initial, relatively sharp, upswing in [³H]muscimol binding, followed by a gradual decrease, closely resembles the pattern of modulation seen in cells transfected with α6β2γ2 GABA_A receptors (Hauser *et al.* 1995). The GABA_A receptor subunit combination of α1β2γ2 has a biphasic pattern of binding as well, but lacks the sharp upswing and gradual decrease seen with the α6β2γ2 combination (Hauser *et al.* 1995). The pattern seen in the bullfrog may thus be due to a combination of α6β2γ2 GABA_A receptors with other GABA_A receptors sensitive to pregnenolone sulfate modulation.

Each subunit from the bullfrog brain had conserved functional domains. The α1-like subunit of the bullfrog contained a conserved region for [³H]muscimol binding, with residues corresponding to the rat α1 subunit amino acids, Tyr-59 to Gln-67, indicating the presence of the [³H]muscimol high-affinity binding site (Smith & Olsen 1994). Also, the bullfrog α1-like subunit possessed a conserved Phe that shared identity with the rat α1 subunit Phe-64, which is covalently modified by [³H]muscimol (Lolait *et al.* 1989, Khrestchatisky *et al.* 1989, Smith & Olsen 1994). However, a leucine at the same position has also been identified in the rat (Sigel *et al.* 1992). Furthermore, the rat Phe-64 and Arg-66, also conserved in the bullfrog, are part of a GABA binding site (Boileau *et al.* 1999). However, the bullfrog subunit lacks identity with the rat α1 subunit Ser-68, and possesses a Gly instead. The Ser-68 of the rat is also part of the rat GABA binding site (Boileau *et al.* 1999). No other α subunit in the rat has a glycine at this position (Tyndale *et al.* 1995). Whether this residue discrepancy influences this particular GABA binding site is not known.

The partial sequence of the bullfrog α1-like subunit contained part of the putative cytoplasmic loop between transmembrane domains M3 and

M4. Like the extracellular region, the intracellular loop represents one of the least conserved regions between subunits. This loop contains phosphorylation sites (Leidenheimer *et al.* 1991). Within this region, two single amino acid residues (rat $\alpha 1$ subunit Asn-350 and Gly-354) are not present in the $\alpha 1$ -like sequence of the bullfrog. The Asn-350 residue is conserved in the rat and chicken, while Gly-354 is only found in the rat. This suggests that mediation of second messenger systems may be less conserved than the ligand binding properties associated with the extracellular region.

The sequence of the bullfrog $\beta 1$ -like subunit revealed conservation of the rat $\beta 1$ subunit Tyr-62 residue associated with high-affinity agonist binding (Newell *et al.* 2000). Also, two GABA binding domains found on the rat $\beta 1$ subunit (as well as β subunits of other phyla) were conserved in the bullfrog $\beta 1$ -like subunit (rat Tyr-157-Thr-160 and Thr-202-Tyr-205; Amin & Weiss 1993). As in the $\alpha 1$ -like subunit, invariant cysteines of the disulfide loop were present on the $\beta 1$ -like partial sequence that shared identity with those at positions 136 and 150 of the rat $\beta 1$ subunit.

The sequences of the bullfrog GABA_A-like subunits extended into regions containing conserved transmembrane domains. All transmembrane domain-spanning regions in the bullfrog GABA_A receptor-like sequences shared 100% identity with the rat transmembrane domains of their subunit counterparts. The M2 transmembrane domain, which was fully sequenced in the bullfrog $\alpha 1$ - and $\gamma 2$ -like subunits, and partially in the $\beta 1$ -like sequence, is composed of several hydrophilic residues and is hypothesized to form the GABA_A receptor ion channel (Salpeter & Loring 1985, Barnard *et al.* 1987). Furthermore, all three of the bullfrog M2 transmembrane domains contained a conserved leucine (rat $\alpha 1$, Leu-263; $\beta 1$, Leu-259; $\gamma 2$, Leu-274) implicated in GABA_A receptor activation (Chang & Weiss 2000). Also, an invariant proline in the M1 transmembrane domain of all three GABA_A-like subunits of the bullfrog was identical to the rat ($\alpha 1$, Pro-232; $\beta 1$, Pro-228; $\gamma 2$, Pro-243). This proline has recently been found to be important in receptor assembly ($\alpha 1$ subunit), barbiturate binding, and linking of GABA binding with channel gating in the $\beta 1$ subunit (Greenfield *et al.* 2002). The complete identity throughout the observed transmembrane domains of the bullfrog GABA_A-like subunit

indicates conserved structural homogeneity of the GABA_A receptor.

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