[3H]RY 80: A High-Affinity, Selective Ligand for γ-Aminobutyric Acid A Receptors Containing Alpha-5 Subunits1, 2

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ABSTRACT

The radiochemical synthesis and pharmacological properties are described of [3H]RY 80 (ethyl-8-acetyl-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxylate, [ethyl-3H]). This compound is one of a series of 8-substituted imidazobenzodiazepines that exhibits both high affinity and selectivity for γ-aminobutyric acid (GABA_A) receptors containing alpha-5 subunits. Saturable, high-affinity (K_d ~0.7 nM) binding of [3H]RY 80 was observed in hippocampal membranes. The maximum number (B_max) of [3H]RY 80 binding sites was ~18% of that obtained with [3H]flunitrazepam, a radioligand that labels all “diazepam-sensitive” GABA_A receptors. This value is consistent with previous estimates (10–20%) of the proportion of rat hippocampal GABA_A receptors containing alpha-5 subunits determined by immunoprecipitation with selective antibodies and competition experiments using an alpha-5-selective ligand. In recombinant GABA_A receptors composed of alpha-5 beta-3 gamma-2 subunits, the K_d of [3H]RY 80 (~0.5 nM) was consistent with the value obtained in hippocampus, whereas the B_max value was not significantly different from that obtained with [3H]flunitrazepam. The potencies of several benzodiazepine site ligands to inhibit [3H]RY 80 binding to hippocampal membranes were in agreement with the values obtained in recombinant (alpha-5 beta-3 gamma-2) GABA_A receptors. This work was supported in part by a predoctoral fellowship from the American Society for Pharmacology and Experimental Therapeutics (C.M.C.) and NIH Grant MH-46851 (J.M.C.). R.J.S. is a PRAT Fellow, NIGMS.

ABBREVIATIONS: GABA, γ-aminobutyric acid; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate; HEK, human embryonic kidney.

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pharmacological heterogeneity of wild-type GABA<sub>A</sub> receptors, which has been recognized for almost 20 years (Klepner et al., 1979; Lippa et al., 1982; Young et al., 1981).

With few exceptions (e.g., GABA<sub>A</sub> receptors in the cerebellum containing alpha-6 subunits, the so-called “diazepam-insensitive GABA<sub>A</sub> receptors”; Gunnersen et al., 1996; Lüddens et al., 1996; Lüddens et al., 1999; Wong et al., 1995), it is inherently more difficult to study the pharmacological properties of benzodiazepine site ligands at specific subpopulations of wild-type compared with recombinant GABA<sub>A</sub> receptors. This is due, in part, to a remarkable receptor heterogeneity present at the cellular level (Fritschy and Mohler, 1995; McKernan and Whiting, 1996; Wisden et al., 1992) and the paucity of high-affinity, selective ligands capable of discriminating among these receptor subpopulations. Based on the ∼10-fold selectivity of Ro 15–4513 for recombinant GABA<sub>A</sub> receptors containing alpha-5 subunits (comparing with receptors containing alpha-1, alpha-2 or alpha-3 subunits); Hadingham et al., 1993; Lüddens et al., 1994), a series of novel 8-substituted imidazobenzodiazepines (Liu et al., 1995, 1996) were prepared in an attempt to increase this selectivity. Several of these compounds exhibited both high-affinity ($K_i$ = 0.4–5 nM) and selectivity (up to 75-fold) for recombinant GABA<sub>A</sub> receptors containing alpha-5 subunits. Moreover, these imidazobenzodiazepines inhibited $[^3H]$flunitrazepam binding to rat hippocampal membranes (that are relatively enriched in GABA<sub>A</sub> receptors containing alpha-5 subunits; McKernan et al., 1991a) with the characteristics of high-affinity, subtype-selective ligands (Liu et al., 1996). The present study describes the radiochemical synthesis and pharmacological properties of one member of this series, $[^3H]$RY 80 (ethyl-8-acetylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4] benzodiazepine-3-carboxylate, [ethyl-$^3$H]). The results obtained in both wild-type and recombinant GABA<sub>A</sub> receptors indicate that $[^3H]$RY 80 is a useful radioligand for studying specific receptor populations containing alpha-5 subunits.

**Materials and Methods**

**Cell culture and transfection.** HEK 293 cells (American Type Culture Collection, Rockville, MD) were maintained at 37°C in 5% CO<sub>2</sub> as previously described (Gunnersen et al., 1996). Cells were transfected with cDNAs for the rat alpha-5, beta-3 and gamma-2 (8, 8 and 5 µg of DNA/10-cm<sup>2</sup> dish containing ∼4 × 10<sup>6</sup> cells) subunits by calcium phosphate precipitation (Gorman et al., 1990). The cells were harvested ∼48 hr later, and a washed membrane suspension was prepared as previously described (Gunnersen et al., 1996). These membrane suspensions were stored at ~70°C until assayed. The beta-3 and gamma-2s cDNAs were subcloned into pcDNA1 and pcDNA3 vectors, respectively (Gunnersen et al., 1996; Harris et al., 1995). The alpha-5 cDNA (the gift of Dr. H. Lüddens, University of Mainz) was subcloned from a BlueScript to a CMV vector by standard techniques.

**Tissue preparation.** Adult male and juvenile (6–8 days postpartum; both sexes) Sprague-Dawley rats (Taconic Farms, Germantown, NY) were killed by decapitation. The brains were rapidly removed and placed in beakers containing ice-cold 50 mM Tris-citrate buffer, pH 7.8. After dissection, the tissues were disrupted in 50 volumes of ice-cold Tris-citrate buffer using a Polytron (20 sec; setting 6–7) (Brinkmann Instruments, Westbury, NY). The homogenates were centrifuged at 20,000 × g (4°C) for 20 min. The supernatants were discarded and the pellets resuspended in an equal volume of buffer and recentrifuged. This “washing” procedure was repeated a total of five times. Tissue suspensions were frozen on solid CO<sub>2</sub> and stored at −70°C until assayed.

**Radioligand binding.** Studies in recombinant receptors were performed in a final volume of 1 ml consisting of: tissue suspension (~0.2 mg of protein), 0.2 M NaCl, $[^3H]$RY 80 or flunitrazepam and 50 mM Tris-citrate buffer, pH 7.8, to volume. For studies in wild-type receptors (from adult hippocampus and juvenile cerebral cortex), the volume of membrane suspension was varied to yield between 0.02 and 0.1 mg of protein/assay. In competition experiments, 50 µl of buffer was replaced by drugs and/or GABA (30 µM); the concentration of $[^3H]$RY 80 routinely used in competition experiments was ~0.5 to 0.6 nM. Nonspecific binding was defined with Ro 15–1788 (10 µM). In pilot experiments to optimize incubation conditions, specific binding of $[^3H]$RY 80 was obtained at a range of temperatures (4°C, 25°C and 37°C), with the optimum ratio of specific and nonspecific binding achieved at 4°C. Under these assay conditions, $[^3H]$RY 80 (0.8 nM) binding to hippocampal membranes reached equilibrium by 30 min and was maintained for ≥2 hr. Samples were routinely harvested at ≥2 hr. Assays (4°C) were terminated after 2 hr by rapid filtration (Brandel M-48R, Gaithersburg, MD) through GF/B filters followed by two 5-ml washes with ice-cold 50 mM Tris-citrate buffer. Radioactivity retained by the filters was measured in an LS 6500 liquid scintillation counter (Beckman Instruments, Palo Alto, CA). Data were analyzed with GraphPad InPlot 4 (GraphPad Software, San Diego, CA). Protein concentrations were determined using the BCA protein assay reagent (Pierce, Rockford, IL).

**In vivo studies.** Adult, male NIH/Swiss mice (~30 g) were injected (0.1 ml i.p.) with graded doses of QHII-066 (7-acetyleno-1,3-dihydro-1-methyl-5-phenyl-2H,1,4-benzodiazepin-2-one or vehicle (10% diluted Emulphor/90% saline). Mice were placed in individual plastic cages and administered either RY-24 [4-buty1-8-acetylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylic acid; 20 mg/kg i.p.] or DMC (7.5 mg/kg i.p.) 10 min. later. Animals were observed (10 min) for the presence of tonic and clonic convulsions (Liu et al., 1996).

**Synthesis of $[^3H]$RY 80.** 8-Acetylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylic acid (15 mg; 0.05 mmol) was added to a 10 ml round-bottom flask and dissolved in N, N-dimethylformamide (DMF) (1 ml). After the addition of 0.2 M lithium hydroxide (0.25 ml), the reaction was stirred at room temperature for 1 hr and then heated (70°C) for 30 min. The reaction mixture was cooled to room temperature, and the solvents were removed under reduced pressure. The residue was taken up in DMF (1 ml) and transferred to a 5-ml tritiation flask. $[^3H]$Ethyl iodide (0.1 mmol) was added to this mixture, and the reaction was stirred at 70°C for 16 hr (fig. 1). The labiles were removed with ethanol, and the residue was taken up in 10 ml of ethanol. Thin-layer chromatography (ethyl acetate/hexanes 10:1) of the crude reaction mixture revealed two major components with $R_f$ values of ~0.4 (RY 80) and ~0.1 (presumed to be the quaternary salt of RY 80). The crude reaction mixture was purified by high performance liquid chromatography on a Zorbax RX-C8 analytical column with a mobile phase of 1% triethylammonium acetate (pH 4.0)/acetonitrile (75:25) at a flow rate of 1 ml/min. The material corresponding to product (detected by UV absorbance at 274 nm) had a retention time of ~25 min. The solvents were removed with multiple ethanol azeotropes, and

![Fig. 1](https://jpet.aspetjournals.org/doi/figure/10.1124/jpet.1997.289.489)
the residue was taken up in 50 ml of ethanol. The specific activity of [ethyl-3H]RY 80 was 55.4 Ci/mmol, as determined by FAB mass spectroscopy. The radiochemical purity of [3H]RY 80 was 99%, as determined by high performance liquid chromatography.

Materials. [3H]Flunitrazepam (specific activity, 85.8 Ci/mmol) was purchased from Dupont-New England Nuclear (Boston, MA). 3-Carbomethoxy-β-carboline, QHII-066 (the 7-acetyleno-congener of diazepam; Huang et al., 1996), RY-24 (the t-butyl ester congener of RY 80; Liu et al., 1996) and 8-acytylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a]benzodiazepine-3-carboxylate were synthesized at the University of Wisconsin-Milwaukee. Ro 15–1788 was donated by Hoffmann-LaRoche (Nutley, NJ). Zolpidem was the gift of Synthelabo (Laboratoire Experimental Recherche Synthelabo; Paris, France). DMCMC was purchased from Research Biochemicals (Natick, MA). All other reagents and chemicals were obtained from standard commercial sources.

Results

[3H]RY 80 binding to recombinant alpha-5 beta-3 gamma-2 receptors and hippocampal membranes. Saturable, high-affinity (K_D, 0.53 ± 0.09 nM) binding of [3H]RY 80 was observed in membranes prepared from HEK 293 cells transfected with cDNAs encoding alpha-5 beta-3 gamma-2 subunits (fig. 2A). The maximum number of binding sites (B_max obtained with [3H]RY 80 (173 ± 9 fmol/mg of protein) was not significantly different from the values obtained using [3H]flunitrazepam (190 ± 12 fmol/mg of protein; K_D, 1.3 ± 0.18 nM) (fig. 2A). The apparent affinity of [3H]RY 80 in hippocampal membranes (K_D, 0.68 ± 0.04 nM vs. 1.5 ± 0.12 nM for [3H]flunitrazepam) was comparable to that obtained in recombinant receptors, whereas the B_max value was ~17.6% of that obtained with [3H]flunitrazepam (302 ± 21 fmol/mg of protein vs. 1712 ± 156 fmol/mg of protein, respectively) (fig. 2B). In identically prepared cerebellar membranes, saturable binding of [3H]RY 80 was not observed using radioligand concentrations of ≤20 nM (data not shown). To confirm that the receptor population labeled by [3H]RY 80 corresponds to GABA_A receptors containing alpha-5 subunits, the effects of several ligands with well-defined characteristics at these receptors were examined. Zolpidem, which binds with low (μM) affinity to recombinant receptors containing alpha-5 beta-3 gamma-2 receptors (Graham et al., 1996; Lüddens et al., 1994; Pritchett and Seeburg, 1990) did not produce a concentration-dependent inhibition of [3H]RY 80 binding to either recombinant alpha-5 beta-3 gamma-2 receptors (fig. 3A) or hippocampal membranes (fig. 3B). In contrast, the potency of an alpha-5 selective ligand, RY-24 (the t-butyl ester congener of RY 80) (Liu et al., 1995), to inhibit [3H]RY 80 binding was similar in recombinant receptors and hippocampal membranes (IC_50, 0.95 ± 0.22 and 0.82 ± 0.13 nM, respectively) (fig. 3). The potency of QHII-066 (the 7-acetyleno congener of diazepam), which exhibits a moderate selectivity for recombinant alpha-5-containing receptors (Huang et al., 1996), was similar in recombinant alpha-5 beta-3 gamma-2 receptors and hippocampal membranes (IC_50, 41 ± 5 vs. 56 ± 10 nM, respectively). GABA increased the potency of QHII-066 to inhibit [3H]RY 80 binding by ~2.5–3-fold in both preparations (IC_50, 17 ± 3 vs. 18 ± 7 nM, respectively) (fig. 3).

Anticonvulsant actions of QHII-066. The anticonvulsant actions of QHII-066 were examined because GABA increased the potency of this compound in vitro (i.e., a positive “GABA-shift”) (fig. 3). Consistent with previous findings (Liu et al., 1996), parenteral administration of DMCMC (7.5 mg/kg) and RY-24 (20 mg/kg) produced tonic and clonic convulsions in 100% and 80% of mice, respectively. Higher doses of RY-24 did not result in a greater percentage of animals exhibiting convulsions (Liu et al., 1996; and data not shown). QHII-066 reduced both RY-24- and DMCMC-induced convulsions in a dose-dependent manner with ED_50 values of ~0.6 and ~2.9 mg/kg, respectively (fig. 4).

[3H]RY 80 binding to neonatal rat cortex. High-affinity (K_D, 0.81 ± 0.25 nM), saturable (B_max, 464 ± 104 fmol/mg of protein) binding of [3H]RY 80 was obtained in cortical membranes prepared from 6- to 8-day-old rat pups (fig. 5). This binding was zolpidem insensitive and inhibited by RY-24 in a concentration-dependent fashion (IC_50, 2.2 ± 0.4 nM) (fig. 5, inset). The B_max value obtained with [3H]RY 80 was ~31% of the value obtained with [3H]flunitrazepam (1461 ± 317 fmol/mg of protein; K_D, 1.2 ± 0.1 nM).

Fig. 2. Saturation studies of: [3H]RY 80 binding to (A) recombinant alpha-5 beta-3 gamma-2 receptors and (B) hippocampal membranes HEK 293 cells were transiently transfected with cDNAs encoding rat alpha-5 beta-3 gamma-2 subunits. The cells were harvested ~48 hr after transfection and membranes prepared as described. Hippocampal membranes were prepared as described. A, Representative saturation isotherm of [3H]RY 80 (●) and [3H]flunitrazepam (●). The K_D and B_max values in these experiments were 0.43 nM and 182 fmol/mg of protein and 1.3 nM and 183 fmol/mg of protein for [3H]RY 80 and [3H]flunitrazepam, respectively. In pilot experiments, the binding of [3H]RY 80 to cerebellar membranes was not saturable using radioligand concentrations of ≤20 nM (data not shown). B, In this representative experiment, the K_D and B_max values were 0.71 nM and 314 fmol/mg of protein and 1.2 nM and 1707 fmol/mg of protein for [3H]RY 80 and [3H]flunitrazepam, respectively. Insets, Roenthal (Scatchard) analysis of the respective saturation isotherms in (A) recombinant tissue and (B) hippocampal membranes. The radioligand concentrations ranged from ~0.12 to 3.7 nM and ~0.5 to 12.6 nM for [3H]RY 80 and [3H]flunitrazepam, respectively. These experiments were repeated three or more times (see Results) with [3H]RY 80 and [3H]flunitrazepam.
Fig. 3. Inhibition of \([^{3}H]\)RY 80 binding to (A) recombinant alpha-5 beta-3 gamma-2 receptors and (B) hippocampal membranes; \([^{3}H]\)RY 80 binding to recombinant and hippocampal membranes was assayed as described. These are representative experiments repeated at least three times. □, QHII-066 + GABA; ○, QHII-066; ★, zolpidem. The x axes depicts the log of concentrations used. The IC_{50} values (in nM) in recombinant and hippocampal membranes were 0.83 and 1.03 for RY-24, 34 and 53 for QHII-066 and 14.3 and 20.9 for QHII-066 + GABA, respectively. The IC_{50} values for zolpidem could not be estimated. Consistent with the described inverse agonist properties of RY 80 demonstrated both in vitro and in vivo (Liu et al., 1995, 1996), GABA (50 μM) inhibited \([^{3}H]\)RY 80 binding by 48.7 ± 4.1% and 41 ± 1.8% in recombinant receptors and hippocampal membranes, respectively. The IC_{50} values calculated for QHII-066 in the presence of GABA reflect this inhibitory effect of GABA. The Hill slopes in recombinant and hippocampal membranes were 0.84 ± 0.1 and 0.72 ± 0.04 for RY-24, 0.99 ± 0.03 and 0.84 ± 0.1 for QHII-066 and 1.05 ± 0.05 and 0.79 ± 0.12 for QHII-066 + GABA, respectively. These experiments were repeated at least three times (see Results).

**Discussion**

The high-affinity and selectivity of several novel imidazobenzodiazepines for recombinant GABA_{A} receptors containing alpha-5 subunits (Liu et al., 1995) suggest that a radiolabeled form of one (or more) of these compounds could be used to examine the pharmacological properties of the corresponding wild-type receptors. Although GABA_{A} receptors containing alpha-5 subunits are minor constituents of the total GABA_{A} receptor pool, both in situ hybridization (Khrestchatisky et al., 1989; Wisden et al., 1992) and immunohistochemical studies (Endo and Olsen, 1993; McKernan et al., 1991a, 1991b; Mertens et al., 1993; Thompson et al., 1992) indicate the rodent hippocampus is relatively enriched in this subunit compared with other brain regions. The feasibility of selectively labeling this receptor subpopulation in hippocampus was supported by competition studies with RY-24, the t-butyl ester congener of RY 80. Thus, RY-24 inhibition of \([^{3}H]\)flunitrazepam binding to hippocampal membranes is best fit to a two-site competition curve, with the high-affinity component (IC_{50} ~ 0.6 nM) representing 16 ± 4% of the sites labeled by \([^{3}H]\)flunitrazepam (Liu et al., 1996). This high-affinity of RY-24 is consistent with both the value obtained in recombinant receptors composed of alpha-5 beta-3 gamma-2 subunits (Liu et al., 1995) and the proportion of these high-affinity sites corresponds to the values obtained by immunoprecipitation with alpha-5 subunit-specific antibodies in rat hippocampus (McKernan et al., 1991a; Mertens et al., 1993). Although other imidazobenzodiazepines in this series exhibit a greater selectivity for recombinant GABA_{A} receptors bearing alpha-5 subunits than RY 80 (~5-fold compared with 60-fold for RY 80; Liu et al., 1995, 1996), this compound was the simplest to prepare in its radiolabeled form (fig. 1).

The binding of \([^{3}H]\)RY 80 to recombinant alpha-5 beta-3 gamma-2 receptors was saturable (fig. 2A), with a K_{d} value (0.53 ± 0.09 nM) comparable to the K_{d} value (~0.5 nM) obtained in recombinant human receptors composed of alpha-5 beta-3 gamma-2 subunits (Liu et al., 1995). Moreover, the B_{max} value obtained with \([^{3}H]\)RY 80 was not significantly different from the value obtained with \([^{3}H]\)flunitrazepam, indicating that both radioligands label the same receptor populations (fig. 2B). Although saturable, high-affinity binding (K_{d}, 0.69 ± 0.07 nM) of \([^{3}H]\)RY 80 was also detected in hippocampal membranes, the B_{max} value was only ~18% of the value obtained with \([^{3}H]\)flunitrazepam, a radioligand thought to label all “diazepam-sensitive” GABA_{A} receptor isoforms. The fraction of hippocampal GABA_{A} receptors labeled by \([^{3}H]\)RY 80 is consistent with the values obtained by both immunoprecipitation with alpha-5-selective antibodies (~15–16%) (Mertens et al., 1993; McKernan et al., 1991a) and competition studies using the alpha-5-selective ligand

Fig. 4. Anticonvulsant actions of QHII-066. Mice were injected (0.1 ml i.p.) with varying doses of QHII-066 or vehicle (5–20 mice/dose). At 10 min later, the mice were injected with RY-24 (20 mg/kg i.p.) or DMCM (7.5 mg/kg i.p.) and observed (10 min) for the presence of convulsions. RY-24 and DMCM produced a maximum of 80% (16 of 20) and 100% (10 of 10) convulsions, respectively. The inability of RY-24 to produce convulsions in 100% of the mice is consistent with previous data (Liu et al., 1996). The ED_{50} values of QHII-066 were 0.6 and 2.9 mg/kg vs. RY-24 and DMCM, respectively.
RY-24 (16%) (Liu et al., 1996). In contrast, saturable binding of [3H]RY 80 (at concentrations of ≤20 nM) was not observed in cerebellar membranes (fig. 2, legend), an observation consistent with both the low expression of alpha-5 subunits in this brain region (McKernan et al., 1991b; Wisden et al., 1992) and the selectivity of RY 80 for recombinant GABA_A receptors bearing this subunit (Liu et al., 1995).

Zolpidem binds with very low (μM) affinity to both recombinant GABA_A receptors containing alpha-5 subunits (Pritchett and Seeburg, 1990; Hadingham et al., 1993 Lüd- dens et al., 1994; fig. 3A) and hippocampal GABA_A receptors that have been immunoprecipitated with alpha-5-selective antibodies (Mertens et al., 1993; McKernan et al., 1991a). Thus, the inability of zolpidem to significantly reduce [3H]RY 80 binding in hippocampal membranes (fig. 3B) is consistent with the hypothesis that this radioligand selectively labels GABA_A receptors bearing alpha-5 subunits. This hypothesis is also consistent with the agreement in potency of RY-24 (an alpha-5-selective ligand) to inhibit [3H]RY 80 binding to hippocampal membranes (fig. 3B) and recombinant GABA_A receptors with alpha-5 subunits (fig. 3A and Liu et al., 1995) and to inhibit a component of [3H]flunitrazepam binding to hippocampal membranes representing ~16% of the total receptor pool (Liu et al., 1996).

The ability of GABA to modulate the affinity of benzodiazepine site ligands (the “GABA shift”) remains a robust neurochemical measure of efficacy. GABA shift assays traditionally use brain membranes (Skolnick et al., 1982) containing heterogeneous receptor populations. The resulting values thus represent an average efficacy because this measure is dependent on subunit composition (Graham et al., 1996; von Blankenfeld et al., 1990). To determine whether ligand efficacy could be correctly predicted in a subpopulation of hippocampal GABA_A receptors using [3H]RY 80, we examined the effect of GABA on QHII-066, the 7-acetyleno congener of diazepam. This compound was recently reported to bind with moderate (≥7-fold) selectivity to recombinant alpha-5 beta-3 gamma-2 receptors compared with isoforms containing other alpha subunits (Huang et al., 1996). GABA produced a ~2.7-fold increase in the potency of QHII-066 in both hippocampal membranes and recombinant alpha-5 beta-3 gamma-2 receptors (fig. 3). If the positive GABA shift obtained with QHII-066 in hippocampal membranes is predictive of in vivo efficacy, then this compound should exhibit some of the pharmacological properties common to other benzodiazepine site agonists. To test this hypothesis, we examined the anticonvulsant properties of QHII-066. This measure was selected because several of the alpha-5-selective imidazobenzodiazepines, such as RY-24, are inverse agonists at alpha-5 beta-3 gamma-2 receptors expressed in Xenopus oocytes (Liu et al., 1995) and are convulsant in mice (Liu et al., 1996). As predicted from its efficacy in hippocampal membranes, QHII-066 blocked RY-24 induced convulsions in a dose-dependent fashion and was ~5-fold less potent against DMCM-induced convulsions (fig. 4). These observations indicate that [3H]RY 80 may be useful in evaluating ligand efficacies at wild-type GABA_A receptors bearing alpha-5 subunits. Although more speculative, the higher potency of QHII-066 in blocking RY-24 compared with DMCM-induced convulsions suggests its anticonvulsant properties may be related to an action at GABA_A receptors containing alpha-5 subunits.

In situ hybridization studies have shown mRNA encoding the alpha-5 subunit is relatively abundant in the neonatal rat brain (Laurie et al., 1992). This expression diminishes substantially during development, and in the adult brain, mRNA encoding the alpha-5 subunit is relatively abundant only in the hippocampus (Wisden et al., 1992). In contrast, either undetectable (McKernan et al., 1991b) or very low levels (Sieghart et al., 1993) of the corresponding protein have been detected with subunit-specific antibodies. Based on a comparison of the B_max values obtained with [3H]RY 80 and flunitrazepam in cortical membranes from 6- to 8-day-old rat pups, GABA_A receptors bearing alpha-5 subunits represent ~31% of the receptor pool (Results and fig. 5). Although it could be argued that [3H]RY 80 is labeling other GABA_A receptor isoforms in the neonatal cortex, its K_I value (0.81 ± 0.25 nM) is similar to that obtained in both adult hippocampus and recombinant receptors (fig. 2). Moreover, [3H]RY 80 binding to juvenile cortex is zolpidem-insensitive and potently inhibited by the alpha-5-selective ligand RY-24 (fig. 5, inset). The apparent discrepancy between the low levels of alpha-5 immunoreactive protein relative to both an abundance of mRNA encoding this subunit and the B_max value estimated with [3H]RY 80 may be attributable to the extensive glycosylation of alpha-5 subunits (Sieghart et al., 1993) that may interfere with the antigen-antibody reaction in neonatal brain.

In summary, [3H]RY 80 appears to label specific populations of GABA_A receptors containing an alpha-5 subunit and may be used in much the same manner as [3H]zolpidem to study receptor populations bearing alpha-1 subunits (De-Vaude and Morrow, 1994). As such, [3H]RY 80 may be used to evaluate the potency and efficacy of compounds at wild-type GABA_A receptors containing alpha-5 subunits, as a radioligands.

Fig. 5. [3H]RY 80 binding to neonatal rat cortex demonstrates a comparison with [3H]flunitrazepam. Cortical membranes were prepared from 6- to 8-day-old rat pups as described in the text. In this representative experiment, (A) the K_I and B_max values for RY 80 (●) and flunitrazepam (■) were 0.62 and 1.5 nM and 322 and 1160 fmol/mg of protein, respectively. B, Rosenthal (Scatchard) plot of the saturation isotherm represented in A. C, [3H]RY 80 binding (0.75 nM) was insensitive to zolpidem (○) but potently inhibited (IC50 = 2.9 nM) by RY-24 (●). The abscissae are in log units. These experiments were repeated three times.
gand for autoradiographic studies and as a probe for examining these receptors after physiological and pharmacological manipulations.

References


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