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Evaluation of native $GABA_A$ receptors containing an $\alpha 5$ subunit

Ming Li, Andras Szabo, Howard C. Rosenberg *

Department of Pharmacology, Medical College of Ohio, 3035 Arlington Ave., Toledo, OH 43614-5804, USA

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Abstract

The type A receptor for γ-aminobutyric acid (GABA), or $GABA_A$ receptor, is a pentamer of highly variable quaternary structure. It includes two α subunits, drawn from a pool of six genes, which largely determine benzodiazepine pharmacology of the receptor. In brain sections, both [^{3}H]RY-80 (ethyl-8-acetylene-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate) and [^{3}H]L-655,708 (ethyl (*S*)-11,12,13,13*a*-tetrahydro-7-methoxy-9-oxo-9*H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*][1,4]benzodiazepine-1-carboxylate), which are selective for the benzodiazepine site of $\alpha 5$ subunit-containing receptors, showed high-affinity, specific binding, but to fewer regions than did the nonselective benzodiazepine, [^{3}H]flunitrazepam. The pattern mirrored $\alpha 5$ mRNA distribution, and was similar to that previously reported for [^{3}H]L-655,708 binding. Displacement of [^{3}H]RY-80 bound to hippocampal homogenates, and of [^{3}H]flunitrazepam bound to cerebellar and hippocampal homogenates showed comparable displacement by flumazenil (K_i's 5–7 nM). However, the K_i's for diazepam and for clobazam to displace [^{3}H]RY-80 binding in hippocampus were about fourfold higher than for [^{3}H]flunitrazepam, and the K_i for clonazepam was sixfold larger, suggesting that these benzodiazepine receptor agonists bind with relatively lower affinity at hippocampal $\alpha 5$-containing receptors. © 2001 Published by Elsevier Science B.V.

Keywords: $GABA_A$ receptor; Autoradiography; [^{3}H]RY-80; [^{3}H]L-655,708; Clonazepam

1. Introduction

The type A receptor for γ-aminobutyric acid (GABA), or $GABA_A$ receptor, is a pentameric ligand-gated Cl^- channel, with several modulatory sites at which various drugs can act, with the benzodiazepine site being the best characterized (Macdonald and Olsen, 1994). The receptor stoichiometry is very complex, with evidence of mammalian genes for up to 6α, 3β and 3γ subunits, as well as δ, ρ, ε, π and θ subunits (Mehta and Ticku, 1999; Whiting, 1999). Benzodiazepine binding and potentiation of the GABA response require the presence of a γ subunit in the $GABA_A$ receptor, especially the γ2 isoform (Gunther et al., 1995; Pritchett et al., 1989). The α subunits of the receptor are also involved in the benzodiazepine recognition site (Sigel and Buhr, 1997; Stephenson et al., 1990) and the particular α subunit composition of a receptor plays a significant role in the benzodiazepine pharmacology of the receptor (Hadingham et al., 1993; Lüddens et al., 1995; Pritchett and Seeburg, 1990). For example, classical benzodiazepines, such as diazepam, do not have significant affinity at receptors that include an $\alpha 4$ or $\alpha 6$ subunit. Agents such as zolpidem have provided a basis for further differentiation of the diazepam-sensitive $GABA_A$ receptors. Zolpidem binds with highest affinity to receptors that include an $\alpha 1$ subunit, but with much less affinity to those with $\alpha 2$ or $\alpha 3$ subunits, and almost not at all to receptors with an $\alpha 5$ subunit. Some data indicated that receptors with both $\alpha 5$ and γ2 subunits constitute the majority of native zolpidem-insensitive benzodiazepine receptors (Lüddens et al., 1994). Selective actions of benzodiazepine ligands on receptors of particular subunit composition could serve as the basis for clinically useful selectivity of drug action, especially if the subunit in question were expressed on a limited number or type of neurons.

The α subunit isoforms of the $GABA_A$ receptor have varying regional patterns of expression, though there are many overlaps. For example, in comparison to the widespread expression of mRNA for the $\alpha 1$ subunit, $\alpha 6$ mRNA is only expressed in cerebellar granule cells (Wisden et al., 1992). The $\alpha 5$ subunit appeared to be expressed primarily in hippocampus and a few other regions, and is absent in many regions, such as the cerebel-

* Corresponding author. Tel.: +1-419-383-4182; fax: +1-419-383-2871.

E-mail address: hrosenberg@mco.edu (H.C. Rosenberg).

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lum (Fritschy and Möhler, 1995; Persohn et al., 1992; Wisden et al., 1992), suggesting that agents selective for α5 subunit-containing $GABA_A$ receptors might have some selectivity of action. In addition, these receptors may be altered in disease states or after experimental treatment. For example, results of studies using the pilocarpine model of chronic seizures suggested that α5 subunit-containing $GABA_A$ receptors may be involved in some types of epilepsy (Houser and Esclapez, 1996; Rice et al., 1996), and one study (Papadimitriou et al., 1998) suggested an association with bipolar affective disorder. In studies of drug tolerance, it has been found that chronic benzodiazepine treatments produce changes in α5 mRNA levels (Impagnatiello et al., 1996; Tietz et al., 1999; Wu et al., 1994a; Zhao et al., 1994) and immunoreactivity (Pesold et al., 1997) which indicate that α5 subunit-containing receptors may be involved in benzodiazepine tolerance or dependence. In fact, recent results (Li et al., 2000) showed regionally restricted down-regulation of benzodiazepine binding involving α5 subunit-containing receptors, which indicated that the hippocampal CA1 region is an important site for adaptive processes during chronic benzodiazepine treatment. The availability of selective ligands for receptors with the α5 subunit provides a useful tool for further studies.

Recently, ligands that appear to be selective for $GABA_A$ receptors containing an α5 subunit were reported. The imidazobenzodiazepine, ethyl-8-acetylene-5,6-dihydro-5-methyl-6-oxo-4 *H*-imidazo[1,5*a*][1,4]benzodiazepine-3-carboxylate (RY-80) appears to have benzodiazepine receptor inverse agonist activity, and produced convulsions in mice (Liu et al., 1996). Another compound, ethyl (*S*)-11,12,13,13*a*-tetrahydro-7-methoxy-9-oxo-9 *H*-imidazo[1,5-*a*]pyrrolo[2,1-*c*][1,4]benzodiazepine-1-carboxylate (L-655,708) was also reported to be quite selective for α5 subunit-containing $GABA_A$ receptors (Quirk et al., 1996). Both were reported to bind selectively, and with high affinity to cells expressing $GABA_A$ receptor α5 subunits along with a β2 or β3 plus a γ2 subunit, and to homogenate preparations of brain tissue from cerebral cortex and hippocampus, but not cerebellum, corresponding to the regional expression of α5 mRNA (Wisden et al., 1992). Moreover, this specific binding could be displaced by nonselective benzodiazepines, such as flunitrazepam, but not by zolpidem (Quirk et al., 1996; Skolnick et al., 1997; Sur et al., 1998). The fraction of hippocampal receptors specifically bound by [^{3}H]RY-80 was similar to that expected from the fraction of hippocampal receptors that could be precipitated with an α5 subunit-selective antibody (Skolnick et al., 1997). The binding of [^{3}H]L-655,708 to rat brain sections was measured, in the presence of zolpidem, using autoradiographic technique, and the distribution was found to be similar to the protein and mRNA distribution for the α5 subunit (Sur et al., 1999).

In the present study, we used the published method for homogenate binding of [^{3}H]RY-80 (Skolnick et al., 1997) to slightly modify the assay for autoradiographic determination of [^{3}H]flunitrazepam binding. This was used for quantitative autoradiographic measurement of [^{3}H]L-655,708 and [^{3}H]RY-80 binding in rat brain, and the patterns of distribution of [^{3}H]L-655,708 and [^{3}H]RY-80 binding sites were compared to each other, and to that of [^{3}H]flunitrazepam, a nonselective benzodiazepine. Additional evaluation of α5-containing $GABA_A$ receptors was done using [^{3}H]RY-80 saturation binding in homogenates prepared from hippocampus, and by displacement assays. A previous study (McKernan et al., 1991) reported that iodoclonazepam bound with relatively low affinity to $GABA_A$ receptors immunoprecipitated with an antibody selective for the α5 subunit. This suggested that the differences between clonazepam and other benzodiazepine receptor agonists, such as diazepam, might be related to relative activity at receptors with, or without an α5 subunit. Assays were done to compare the displacement of [^{3}H]flunitrazepam binding to hippocampal and cerebellar homogenates with the displacement of [^{3}H]RY-80 binding to hippocampal homogenates by the nonselective benzodiazepine receptor antagonist, flumazenil, the prototype benzodiazepine, diazepam, and two benzodiazepines with some selectivity as anticonvulsants, clonazepam and clobazam.

2. Materials and methods

Male Sprague–Dawley rats (250–300 g) were used for these experiments. All procedures were approved by the Institutional Animal Care and Use Committee of the Medical College of Ohio, and conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

2.1. Autoradiographic binding assay

2.1.1. Slide preparation

After decapitation, the brains were quickly removed and immersed in isopentane cooled in an acetone-dry ice bath, then stored at −70°C in air-tight vials. Coronal sections, 10 μm thick, were prepared at −14°C using a microtome, and thaw-mounted onto slides (coated with 0.5% gelatin and 0.05% chrome alum). The slides were then transferred to ice-cold slide boxes and stored at −70°C until the time of the binding assay. A few sections were saved for Cresyl violet staining to facilitate identification of brain regions.

2.1.2. [^{3}H]benzodiazepine binding

The [^{3}H]RY-80 assay was based on a standard assay for [^{3}H]flunitrazepam (described below), but slightly modified based on the reported method for [^{3}H]RY-80 binding to tissue homogenates (Skolnick et al., 1997). The same assay was then employed for [^{3}H]L-655,708. After removing the slide-mounted brain sections from the freezer, they were

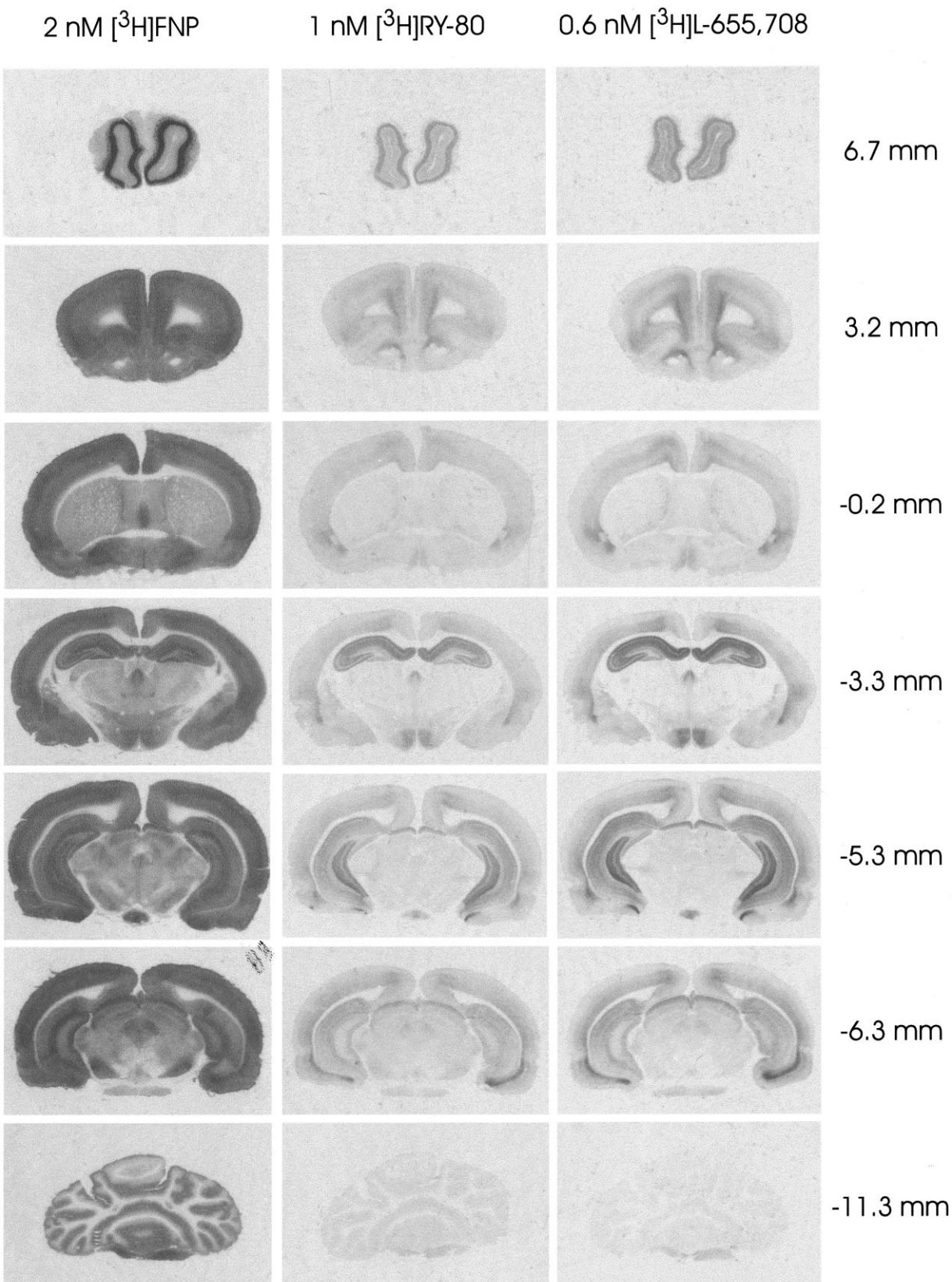

Fig. 1. Representative autoradiographs showing the binding of 2.0 nM [^{3}H]flunitrazepam (FNP), 1.0 nM [^{3}H]RY-80 and 0.6 nM [^{3}H]L-655,708 to coronal sections of rat brain. The numbers on the right indicate distance, in mm, from bregma, according to Paxinos and Watson (1986).

rapidly dried with a stream of cold air, then pre-washed four times for a total of 30 min at 0°C in 50 mM Tris-citrate, pH 7.8, in 0.2 M NaCl. Following this, slides used for studying the regional distribution of binding were incubated with 1 nM [^{3}H]RY-80 (55 Ci/mmol; New England Nuclear, Boston, MA) or 0.6 nM [^{3}H]L-655,708 (83 Ci/mmol; Amersham, Arlington Heights, IL) at 0°C for 1 h. Concentrations were selected to be near the reported K_d in tissue homogenates (Skolnick et al., 1997; Sur et al., 1998). Nonspecific binding to adjacent tissue sections was determined in the presence of 1 μM flumazenil. Slides used to investigate the concentration-dependence of binding were incubated with 0.1–30 nM [^{3}H]RY-80 or [^{3}H]L-655,708. The incubation was terminated by rinsing twice for 30 s in ice-cold 50 mM Tris-citrate, pH 7.8, in 0.2 M NaCl. The slides were then dipped briefly in cold distilled water and dried with a stream of cool air. Slide-mounted tissue sections for autoradiography were fixed with paraformaldehyde vapor at 80°C, and exposed to tritium-sensitive film for 1–5 weeks (according to the ligand concentration used). In preliminary studies, sections were incubated for various lengths of time (10 min–2 h) to determine the time course for equilibration. After the incubation, and following the washing and rapid drying steps, sections were scraped into scintillation vials using a cotton swab moistened with ethanol, 10 ml scintillation fluid (CytoScint; ICN, Costa Mesa, CA) was added, and radioactivity was counted. For both [^{3}H]RY-80 and [^{3}H]L-655,708 specific binding, equilibrium was essentially complete by 30 min, and the binding remained stable at least up to 2 h.

For [^{3}H]flunitrazepam binding, brain sections were pre-incubated in 0.17 M Tris–HCl buffer (pH 7.4) at 0°C for 30 min. Sections were then incubated in Tris–HCl buffer containing 2 nM [^{3}H]flunitrazepam (85 Ci/mmol; New England Nuclear) for 1 h at 0°C. Nonspecific binding was determined in the presence of 2 μM clonazepam. Incubation was terminated by washing the sections twice (30 s each) in ice-cold 0.17 M Tris–HCl buffer, followed by a brief rinse in ice-cold distilled water. Finally, the slides were dried with a stream of cold air, and fixed with paraformaldehyde vapor at 80°C, then exposed to tritium-sensitive film for 2 weeks.

2.1.3. Data analysis

After developing the exposed film, the images were digitized, and ligand binding was quantified with computer-assisted densitometry using the NIH Image software. To quantify optical density over particular brain regions, the optical densities of co-exposed standards were determined, and a standard curve was generated. The tritium standards were 10 disks of rat brain paste mounted on a single slide which contained known amounts of [^{3}H]thymidine (Tietz et al., 1986). The different brain regions were identified using a rat brain atlas (Paxinos and Watson, 1986), and by comparing the images to those of Cresyl violet-stained sections. Brain sections from five rats were used to study the regional distribution of specific binding by each radioligand, and sections from three rats were used for evaluating the concentration-dependence of specific binding. The value for optical density over each area of interest was taken as the average of the values determined from three sections prepared from the same rat. Specific binding was determined by subtracting nonspecific binding from total binding, and by converting optical density measurement to pmol/mg protein.

2.2. Homogenate binding assay

Membrane homogenates were prepared as described previously (Wu et al., 1994b). After decapitation, rat hippocampus and cerebellum were collected and homogenized in 0.32 M sucrose. The homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was re-centrifuged at $20{,}000 \times g$ for 20 min. The resulting pellet was washed two more times with 50 mM Tris-citrate, pH 7.8, by re-suspension followed by centrifugation. The pellet was finally re-suspended in 0.2 M NaCl containing 50 mM Tris-citrate, pH 7.8.

Binding was performed by incubating hippocampal membrane homogenates (0.1–0.2 mg protein/ml) with [^{3}H]RY-80 or [^{3}H]flunitrazepam in 50 mM Tris-citrate, pH 7.8, in 0.2 M NaCl for 60 min at 4°C. In initial experiments, hippocampal membranes were incubated with 0.2–8 nM [^{3}H]RY-80, using 1000-fold excess flumazenil to determine nonspecific binding, and the B_{max} and K_d values of specific binding were calculated. Additional experiments were done to evaluate the binding of 2 nM [^{3}H]RY-80 to hippocampal membranes, the binding of 2 nM

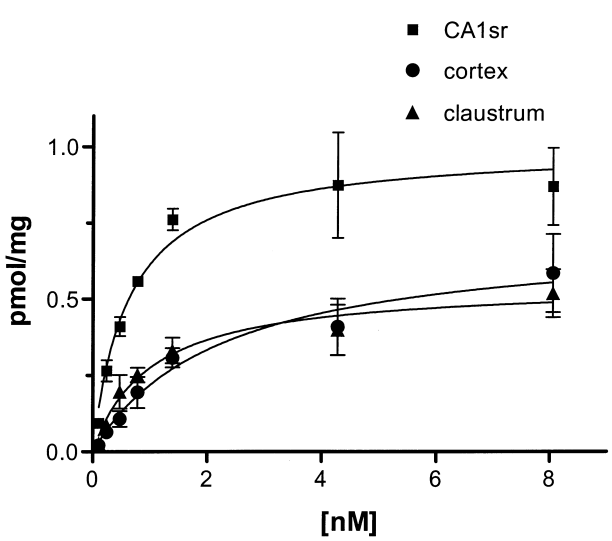

Fig. 2. Concentration-dependence of [^{3}H]L-655,708 specific binding to rat brain sections ($n = 3$). Three representative regions are shown: stratum radiatum of the hippocampal CA1 region, lamina VI of the motor cortex (hindlimb region), and the claustrum.

[^{3}H]flunitrazepam to hippocampal and cerebellar membrane homogenates, and the displacement of this binding by other benzodiazepines. The radioligands were incubated in the absence or in the presence of clonazepam (1 nM–10

Table 1
Distribution of [^{3}H]RY-80, [^{3}H]L-655,708 and [^{3}H]FNP specific binding

Brain region	[^{3}H]FNP	[^{3}H]RY-80	[^{3}H]L-655	RY/FNP	L655/FNP
Olfactory bulb					
External plexiform layer	2483 ± 99	209 ± 39	177 ± 28	0.08	0.07
Glomerular layer	1009 ± 49	61 ± 14	78 ± 12	0.06	0.08
Claustrum	1215 ± 48	58 ± 11	56 ± 8	0.05	0.05
Tenia tecta	882 ± 57	30 ± 6	18 ± 3	0.03	0.02
Dorsal endopiriform nucleus	885 ± 43	187 ± 32	215 ± 30	0.21	0.24
Frontoparietal cortex, motor area					
Lamina I, II, III	1350 ± 55	31 ± 5	22 ± 4	0.02	0.02
Lamina IV	1590 ± 38	85 ± 12	96 ± 16	0.05	0.06
Lamina V	1411 ± 45	50 ± 7	56 ± 7	0.04	0.04
Lamina VI	1144 ± 55	77 ± 14	94 ± 8	0.07	0.08
Frontoparietal cortex, somatosensory area					
Lamina I, II, III	1192 ± 83	24 ± 5	12 ± 4	0.02	0.01
Lamina IV	1437 ± 96	52 ± 11	51 ± 6	0.04	0.04
Lamina V	1209 ± 81	51 ± 11	50 ± 8	0.04	0.04
Lamina VI	1177 ± 80	58 ± 12	71 ± 10	0.05	0.06
Entorhinal cortex					
Lamina I, II, III	1186 ± 49	56 ± 16	61 ± 15	0.05	0.05
Lamina IV	1303 ± 43	122 ± 35	154 ± 34	0.09	0.12
Lamina V	1401 ± 65	124 ± 24	150 ± 27	0.09	0.11
Lamina VI	1671 ± 76	385 ± 71	505 ± 78	0.23	0.30
Caudate–putamen	490 ± 36	7 ± 2	4 ± 1	0.01	0.01
Medial preoptic area	866 ± 63	52 ± 9	69 ± 13	0.06	0.08
Hippocampal formation					
CA1, stratum oriens	1368 ± 61	448 ± 67	610 ± 74	0.33	0.45
CA1, stratum pyramidale	1166 ± 30	328 ± 60	391 ± 59	0.28	0.33
CA1, stratum radiatum	1450 ± 43	478 ± 69	599 ± 71	0.33	0.41
CA3, stratum oriens	1032 ± 55	220 ± 38	307 ± 37	0.21	0.30
CA3, stratum pyramidale	790 ± 28	90 ± 19	114 ± 17	0.11	0.14
CA3, stratum radiatum	1127 ± 57	248 ± 45	312 ± 31	0.22	0.28
Dentate gyrus, molecular layer	1616 ± 56	129 ± 25	153 ± 20	0.08	0.09
Dentate gyrus, granular layer	906 ± 32	36 ± 6	35 ± 5	0.04	0.04
Subiculum	1032 ± 81	118 ± 22	142 ± 15	0.11	0.14
Amygdala					
Lateral nucleus	1452 ± 25	50 ± 10	38 ± 7	0.03	0.03
Basolateral nucleus	1209 ± 22	54 ± 11	68 ± 9	0.04	0.06
Medial postero-dorsal nucleus	829 ± 57	30 ± 7	24 ± 5	0.04	0.03
Medial postero-ventral nucleus	1203 ± 62	78 ± 8	87 ± 13	0.06	0.07
Thalamus					
Posterior paraventricular nucleus	1112 ± 66	110 ± 22	129 ± 25	0.10	0.12
Central medial nucleus	738 ± 14	11 ± 2	5 ± 1	0.01	0.01
Ventral posterolateral nucleus	309 ± 2	5 ± 2	1 ± 1	0.02	0.00
Hypothalamus					
Ventromedial nucleus	1243 ± 61	202 ± 33	272 ± 44	0.16	0.22
Arcuate nucleus	474 ± 58	51 ± 12	54 ± 9	0.11	0.11
Substantia nigra					
pars compacta	590 ± 67	11 ± 2	5 ± 1	0.02	0.01
pars reticulata	1003 ± 121	14 ± 4	3 ± 1	0.01	0.00
Medial mammillary nucleus	1564 ± 103	129 ± 25	186 ± 37	0.08	0.12
Superior colliculus					
Superficial gray layer	1833 ± 68	172 ± 25	222 ± 24	0.09	0.12
Optic layer	1367 ± 46	60 ± 13	66 ± 15	0.04	0.05
Intermediate gray layer	1262 ± 19	36 ± 7	31 ± 5	0.03	0.02
Pons, frontal portion	285 ± 60	20 ± 9	34 ± 11	0.07	0.12
Cerebellum					
Granular layer	292 ± 30	8 ± 2	2 ± 1	0.03	0.01
Molecular layer	933 ± 48	16 ± 3	4 ± 1	0.02	0.00

Coronal sections were incubated with 1 nM [^{3}H]RY-80, 0.6 nM [^{3}H]L-655,708, or 2 nM [^{3}H]FNP. Nonspecific binding was determined by incubation in the presence of excess flumazenil. Values are mean ± S.E.M. of five brains (each data point was averaged from three sections) in fmol/mg protein.

μM), flumazenil (1 nM–10 μM), diazepam (1 nM–10 μM), or clobazam (0.1–1000 μM). The reaction was terminated by the addition of 5 ml ice-cold 50 mM Tris-citrate, pH 7.8, in 0.2 M NaCl, and rapid filtration under controlled suction through glass fiber filters (no. 32; Schleicher and Schuell, Keene, NH). The filters were washed two more times with 5 ml ice-cold 50 mM Tris-citrate, pH 7.8, in 0.2 M NaCl, and were allowed to equilibrate overnight in CytoScint scintillation cocktail before counting. The binding data were evaluated using GraphPad Prism software (GraphPad Software, San Diego, CA).

3. Results

Under the assay conditions used, all radioligands provided good autoradiographs with clear, regionally specific binding (Fig. 1). [^{3}H]Flunitrazepam binding was quite similar to published data, and the distribution of [^{3}H]L-655,708 largely confirmed the results obtained using a similar technique for the binding of [^{3}H]L-655,708 in the presence of zolpidem (Sur et al., 1999). Nonspecific binding of [^{3}H]RY-80 and of [^{3}H]L-655,708 were quite low, being similar to film background even with the highest concentrations used. Nonspecific binding of [^{3}H]flunitrazepam was similarly low. Both [^{3}H]RY-80 and [^{3}H]L-655,708 showed binding over many brain areas known to have benzodiazepine receptors (e.g., the hippocampal formation). However, there was an obvious regional heterogeneity of binding, with different patterns for the nonselective [^{3}H]flunitrazepam and the α5 subunit-selective ligands. Many areas densely labeled by [^{3}H]flunitrazepam, such as the substantia nigra and cerebellum, had very low binding with either of the two α5 subunit-selective ligands.

Both [^{3}H]RY-80 and [^{3}H]L-655,708 showed concentration-dependent binding (Fig. 2). [^{3}H]L-655,708 and [^{3}H]RY-80 specific binding in brain areas where α5 is abundant, such as hippocampus, appeared to be mostly to a high-affinity site, but with some contribution from a lower affinity site. Attempts to evaluate these data showed a significantly better fit for two sites. However, for both ligands, these calculations were obviously not reliable, presumably due to the low affinity binding at the higher ligand concentrations. To evaluate the binding, the data in Fig. 2 were analyzed by nonlinear regression, using only [^{3}H]L-655,708 concentrations lower than 10 nM. This analysis resulted in calculated values for B_{max} (pmol/mg protein) and K_d (nM) as follows (mean ± S.E.M., $n = 3$): hippocampal CA1 region, stratum radiatum, $B_{max} = 1.00 \pm 0.07$; $K_d = 0.62 \pm 0.07$; cerebral cortex, lamina VI, $B_{max} = 0.71 \pm 0.11$; $K_d = 2.25 \pm 0.91$; claustrum, $B_{max} = 0.55 \pm 0.06$; $K_d = 1.04 \pm 0.30$. Though values could be obtained for the high affinity site, the values for the lower affinity site were wildly divergent among samples, suggesting that the incubation and washing procedures used in the binding assay with tissue sections did not allow an accurate determination of such low affinity specific binding at this site. Without an accurate determination for this low affinity site, the calculated values for the higher affinity site should probably be considered only as estimates.

To compare the regional distributions of [^{3}H]RY-80, [^{3}H]L-655,708 and [^{3}H]flunitrazepam binding, assays were performed in coronal sections taken at eight levels (Fig. 1). Overall, both α5 subunit-selective ligands appeared to label brain regions with a similar relative intensity. Based on this binding, several areas were chosen for analysis, along with a few other selected areas of interest, such as the cerebellum. These results are presented in Table 1. For both [^{3}H]RY-80 and [^{3}H]L-655,708, the highest binding density was over the hippocampal formation. In particular, the signal was strongest over the CA1 stratum oriens and stratum radiatum. Binding was slightly less intense over the CA3 region and the dentate region. The frontoparietal cortex had moderate labeling, though it might be noted that, in relation to the other cortical lamina, lamina VI was relatively more prominent than with [^{3}H]flunitrazepam binding. In the entorhinal cortex, both [^{3}H]RY-80 and [^{3}H]L-655,708 binding were very pronounced, especially over lamina VI. Several other areas, including the olfactory bulb, claustrum, dorsal endopiriform nucleus, amygdala, hypothalamic nuclei, medial mammillary nucleus, and superficial gray layer of the superior colliculus, had moderate binding. A clear regional specificity of these ligands was evident in several regions. For example, the posterior paraventricular nucleus of the thalamus had quite clear labeling, while other thalamic nuclei were essentially not labeled. Other areas that had little or no labeling by [^{3}H]RY-80 or [^{3}H]L-655,708 included the substantia nigra, caudate–putamen and the pons. In the cerebellum, there

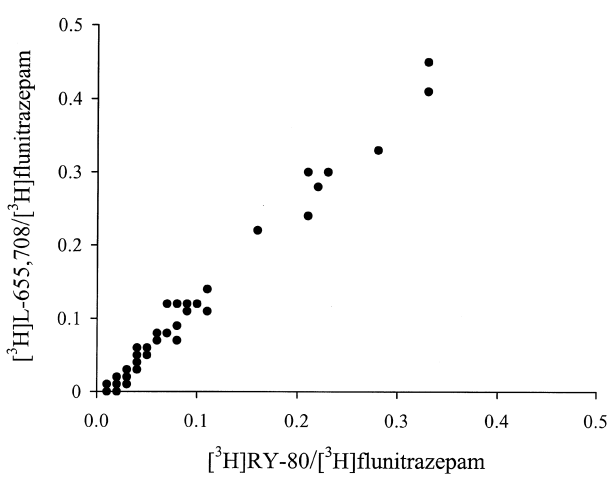

Fig. 3. Relationship between [^{3}H]RY-80 and [^{3}H]L-655,708 binding in rat brain. Each point was plotted based on the values from one brain region, as shown in Table 1. For each ligand, the relative binding density was normalized to the binding density for the non-selective benzodiazepine receptor ligand, [^{3}H]flunitrazepam in each brain region.

Table 2
Displacement of 2 nM [^{3}H]RY-80 and 2 nM [^{3}H]flunitrazepam specific binding

Displacer	[^{3}H]FNP, cerebellum		[^{3}H]FNP, hippocampus		[^{3}H]RY-80, hippocampus		K_i ratio[a]
	K_i (nM)[b]	n_H (range)[c]	K_i (nM)	n_H (range)	K_i (nM)	n_H (range)	
flumazenil	5.3 ± 0.4	1.08–1.22	4.9 ± 0.6	0.99–1.30	7.4 ± 0.1	0.71–0.90	1.5
clonazepam	2.5 ± 0.1	1.0–1.24	5.6 ± 0.5	0.91–1.13	32.6 ± 4.2	0.61–0.68	5.8
diazepam	28.2 ± 1.5	0.77–0.92	21.5 ± 6.7	0.58–0.81	80.9 ± 3.1	0.67–0.99	3.8
clobazam	924 ± 62	0.96–1.12	899 ± 50	0.68–1.04	3787 ± 442	0.82–0.98	4.2

[a]Ratio of the k_i value for displacing [^{3}H]RY-80 binding in hippocampal tissue, compared to the k_i value for displacing [^{3}H]flunitrazepam binding in hippocampal tissue.
[b]mean ± S.E.M. $n = 3$.
[c]Range of Hill slopes.

was virtually no signal with [^{3}H]L-655,708. However, there was slight labeling with [^{3}H]RY-80, especially over the granule cell layer, which was most noticeable with higher ligand concentrations.

To allow a direct comparison of the regional binding densities of [^{3}H]RY-80 and [^{3}H]L-655,708, the values were normalized to the binding of the nonselective ligand, [^{3}H]flunitrazepam, in each brain region by expressing the binding as a ratio to that of flunitrazepam. These ratios, shown in Table 1, were used to correlate the relative regional densities of binding by the selective ligands. The results of this correlation are shown in Fig. 3. There was a clear linear correlation ($r = 0.99$; $P < 0.001$) between [^{3}H]RY-80 and [^{3}H]L-655,708 binding densities, with no region deviating from this relationship.

The properties of $\alpha 5$ subunit-containing receptors were further evaluated in hippocampal homogenates using [^{3}H]RY-80. In the concentration range of 0.2–8 nM, [^{3}H]RY-80 specific binding in hippocampal tissue was saturable, with high affinity ($K_d = 2.2 \pm 0.10$ nM), and with a lower B_{max} (0.61 ± 0.06 pmol/mg) than typically found with [^{3}H]flunitrazepam. Several benzodiazepines were compared for the ability to displace specifically bound [^{3}H]flunitrazepam and [^{3}H]RY-80 (Table 2). The benzodiazepine receptor antagonist, flumazenil, displaced specifically bound 2 nM [^{3}H]flunitrazepam with high potency in tissues from hippocampus ($K_i = 4.9$ nM) and cerebellum ($K_i = 5.3$ nM), and had a very similar high potency to displace specifically bound 2 nM [^{3}H]RY-80 from hippocampus ($K_i = 7.4$ nM). In sharp contrast, using clonazepam as the displacer revealed a striking difference (Fig. 4). In cerebellar tissue, clonazepam readily displaced [^{3}H]flunitrazepam ($K_i = 2.5$ nM). In hippocampal tissue, clonazepam had a slightly lower potency to displace [^{3}H]flunitrazepam ($K_i = 5.6$ nM). However, clonazepam displacement of [^{3}H]RY-80 binding to hippocampal membranes showed a much lower apparent affinity ($K_i = 32.6$ nM). As expected, diazepam was somewhat less potent than clonazepam (Table 2). Diazepam displaced [^{3}H]flunitrazepam bound to hippocampal and cerebellar membranes with similar potency, but had a three- to fourfold lower potency for displacing [^{3}H]RY-80 bound to hippocampal membranes. The 1,5-benzodiazepine, clobazam, was of even lower potency, and produced a similar pattern as did diazepam (Table 2). Clobazam displaced specifically bound [^{3}H]flunitrazepam from hip-

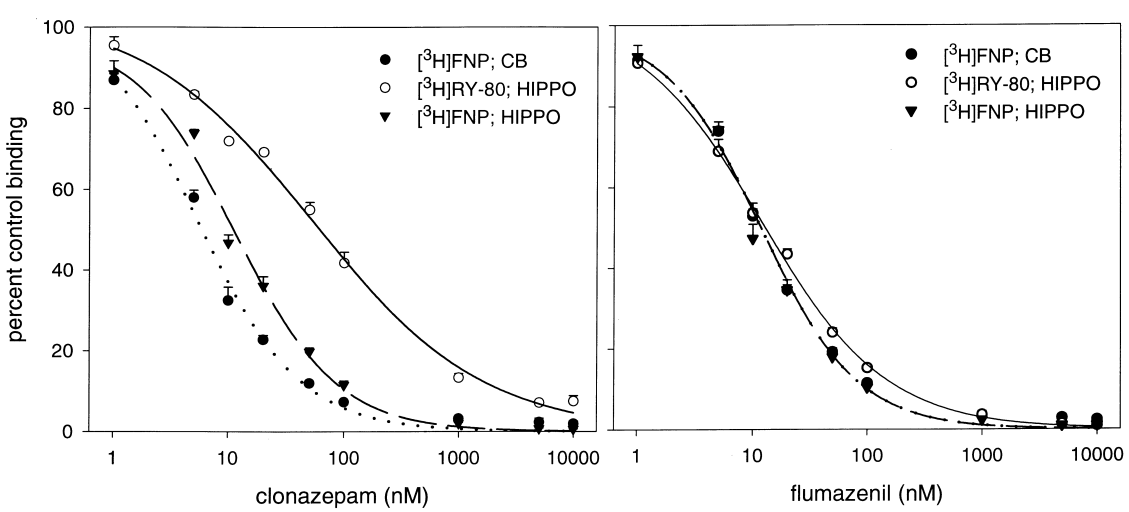

Fig. 4. The displacement of [^{3}H]RY-80 and [^{3}H]flunitrazepam (FNP) binding by 1–10,000 nM clonazepam or flumazenil ($n = 3$). Hippocampal (HIPPO) or cerebellar (CB) membrane homogenates were incubated with 2 nM [^{3}H]RY-80 or 2 nM [^{3}H]FNP with or without the displacer drug.

pocampal and cerebellar membranes with similar potency, but had a fourfold lower potency for displacing [^{3}H]RY-80 binding to hippocampal membranes.

4. Discussion

Studies of [^{3}H]RY-80 and [^{3}H]L-655,708 binding in cells expressing recombinant $GABA_A$ receptors and in brain homogenates have indicated that these ligands are selective for $GABA_A$ receptors containing an $\alpha 5$ subunit (Liu et al., 1996; Quirk et al., 1996; Skolnick et al., 1997; Sur et al., 1998). In the present study, the regional binding of these radioligands supported the selectivity of binding to $\alpha 5$ subunit-containing $GABA_A$ receptors. Both [^{3}H]RY-80 and [^{3}H]L-655,708 binding showed greater regional heterogeneity than did the nonselective benzodiazepine, [^{3}H]flunitrazepam. The pattern was consistent with the results of in situ hybridization studies (Khrestchatisky et al., 1989; Persohn et al., 1992; Tietz et al., 1999; Wisden et al., 1992) and immunohistochemical studies (Fritschy and Möhler, 1995), with the highest [^{3}H]RY-80 and [^{3}H]L-655,708 binding density over the hippocampal region. Other regions reported to express $\alpha 5$ mRNA (Persohn et al., 1992; Wisden et al., 1992) generally showed corresponding [^{3}H]RY-80 and [^{3}H]L-655,708 binding. For example, the studies cited above indicated expression in cerebral cortex, with a relatively more intense signal for $\alpha 5$ mRNA within cortical lamina VI, which corresponded to [^{3}H]RY-80 and [^{3}H]L-655,708 binding over this layer. It would be of interest to know if there is a particularly high $\alpha 5$ mRNA expression in lamina VI of the entorhinal cortex to correspond to the higher density of $\alpha 5$ selective labeling in this cerebral cortical region as compared to the frontoparietal region (Table 1). Other areas found to have good $\alpha 5$ subunit-selective binding and which were reported to show mRNA expression by in situ hybridization included the superficial layer of the superior colliculus, hypothalamic nuclei, the claustrum and the olfactory bulb. Of equal importance was the lack of binding in areas that were reported as not expressing $\alpha 5$ mRNA. An interesting example is that of the thalamus, where only the area corresponding to the posterior paraventricular nucleus was found to have [^{3}H]RY-80 and [^{3}H]L-655,708 specific binding. Of all the thalamic areas evaluated, this was the only one with a detectable signal for $\alpha 5$ mRNA (Wisden et al., 1992).

As a function of concentration, [^{3}H]RY-80 and [^{3}H]L-655,708 both appeared to be quite selective. Results of varying the concentration suggested saturation of a high-affinity site, as well as binding to a lower-affinity site, or sites. However, since the binding at higher ligand concentrations was displaced by excess unlabeled flumazenil, it still represented specific binding, though of much lower affinity, apparently to a wider variety of $GABA_A$ receptors than only those that included an $\alpha 5$ subunit. This selectivity was in keeping with the relative affinities of [^{3}H]RY-80 (Liu et al., 1996) and [^{3}H]L-655,708 (Quirk et al., 1996) at recombinant receptors of varying composition. One difference between the two $\alpha 5$ subunit-selective ligands was noted especially with the highest concentrations, at which [^{3}H]RY-80 appeared to faintly label the cerebellum. This may be related to lower-affinity binding to $GABA_A$ receptors containing $\alpha 6$ subunits. In recombinant receptors, the relative selectivity of [^{3}H]RY-80 for $\alpha 5$ subunit-containing receptors was lowest compared to receptors expressed with $\alpha 6$ rather than another α variant (Liu et al., 1996). Though both radioligands proved useful for autoradiographic binding assays, with the particular assay conditions employed in this study, [^{3}H]L-655,708 produced a slightly better image that allowed easier identification of regional boundaries.

The results of the present study may be compared to the [^{3}H]L-655,708 binding presented previously (Sur et al., 1999). In that study, there were some small differences in the assay conditions, plus zolpidem was used to prevent association with $GABA_A$ receptors not containing an $\alpha 5$ subunit. The major observation was that the localization of binding and the relative binding densities in that study were quite comparable to the results of the present work. In comparison to the methods used here, inclusion of zolpidem in the assay did allow a more accurate determination of saturation binding parameters to the high affinity site in the autoradiographic assay. However, for studies in which a treatment can have differential effects on various $GABA_A$ receptor populations, use of both unlabeled zolpidem and the $\alpha 5$ subunit-selective ligand may provide spurious results. In a previous work from this laboratory, it was found that benzodiazepine tolerance may be associated with a selective loss of [^{3}H]zolpidem binding, even in regions and at time points when there is no overall loss of benzodiazepine binding, as determined with [^{3}H]flunitrazepam (Wu et al., 1994b; Wu et al., 1995). Thus, though including zolpidem in the assay may allow accurate determination of binding parameters, it would present an additional variable in studies in which an experimental treatment may alter zolpidem binding, as was found in studies using benzodiazepine tolerant rats.

Selective benzodiazepine ligands, especially those selective for $GABA_A$ receptors that have a limited distribution, could prove to be useful therapeutic agents. They are also useful experimental tools which have already helped define $GABA_A$ receptor types. One useful classification of $GABA_A$ receptors is based on their affinity for benzodiazepine ligands that have relative selectivities for receptors incorporating various α subunits. In particular, zolpidem has proven useful for studying receptors with an $\alpha 1$ subunit. However, zolpidem binding also depends on other subunits as well, and it appears that the presence of a $\gamma 3$ subunit also causes the receptor to be insensitive to zolpidem (Lüddens et al., 1994). It has been suggested that

native zolpidem-insensitive $GABA_A$ receptors are largely comprised of receptors that include an $\alpha 5$ and a $\gamma 2$ subunit (Hadingham et al., 1993; Lüddens et al., 1995; Pritchett and Seeburg, 1990). If this were so, there should be an inverse relationship between the relative binding of $\alpha 1$- and $\alpha 5$ subunit-selective ligands. This idea was explored by comparing the ratio of [^{3}H]L-655,708 to [^{3}H]flunitrazepam binding (Table 1), to the ratio of [^{3}H]zolpidem to [^{3}H]flunitrazepam binding presented by Niddam et al. (1987) in 24 areas in which these data were available. This analysis showed a moderate negative correlation (Pearson $r = -0.48$; $P < 0.02$). It should be noted that this excludes many brain regions to which zolpidem binds (Niddam et al., 1987), but which were not evaluated in the present study due to very low binding of the $\alpha 5$ subunit-selective ligands. Even so, comparing the findings of the present study with the regional distribution of [^{3}H]zolpidem binding generally supports the proposal that $\alpha 5$ subunit-containing $GABA_A$ receptors account for a large proportion of zolpidem-insensitive sites, though receptors containing other α subunits, or $\gamma 3$ subunits (Lüddens et al., 1994) must also play a role.

In hippocampal homogenates, [^{3}H]RY-80 binding was similar to that reported by others (Skolnick et al., 1997). In the displacement assays, the nonselective benzodiazepine receptor antagonist, flumazenil, had a similar potency to inhibit [^{3}H]RY-80 and [^{3}H]flunitrazepam binding to hippocampal membranes and [^{3}H]flunitrazepam binding to cerebellar homogenates. In hippocampal tissue, which is relatively enriched in $\alpha 5$ subunit-containing receptors, any difference in displacement of [^{3}H]RY-80 and [^{3}H]flunitrazepam binding could be indicative of selectivity related to the presence of the $\alpha 5$ subunit. Similarly, as cerebellar tissue is essentially devoid of $\alpha 5$ expression (and [^{3}H]RY-80 binding), displacement of binding would be a measure of the displacing drug's action at $GABA_A$ receptors that did not include an $\alpha 5$ subunit. Based on the displacement results, flumazenil had no apparent selectivity. The displacement assay was also used to evaluate the possibility that clonazepam might have a relative selectivity for receptors other than those that include an $\alpha 5$ subunit. This idea was based on the observation (McKernan et al., 1991) that receptors immunopurified with an $\alpha 5$ subunit-selective antibody had very low affinity for [^{125}I]iodoclonazepam, while receptors immunopurified with antibodies selective for $\alpha 1$, $\alpha 2$ or $\alpha 3$ subunits bound this same ligand with K_d's less than 1 nM. In the competition assay (Fig. 4), clonazepam had a much lower potency for displacing [^{3}H]RY-80 binding than for [^{3}H]flunitrazepam binding, and a much lower potency for inhibiting [^{3}H]RY-80 binding than did flumazenil. This pattern supported the hypothesis that clonazepam has a relative selectivity for $GABA_A$ receptors that do not include an $\alpha 5$ subunit. Though diazepam and clobazam showed less selectivity to displace the bound drug from non-$\alpha 5$ containing receptors, the data did indicate some selectivity.

These findings indicate that both [^{3}H]RY-80 and [^{3}H]L-655,708 are useful tools for studying $\alpha 5$ subunit-containing $GABA_A$ receptors, using either homogenate binding assays or quantitative autoradiographic assay. The distribution of binding, in comparison to the binding of other ligands, supports the specificity of binding, and also indicates that $GABA_A$ receptors with an $\alpha 5$ subunit constitute a significant proportion of receptors that do not bind zolpidem. By studying the inhibition of [^{3}H]RY-80 and [^{3}H]flunitrazepam binding, it was shown that clonazepam has a particularly low potency at $GABA_A$ receptors that include an $\alpha 5$ subunit. This may be related to the relative selectivity of clonazepam as an anticonvulsant (Homan and Rosenberg, 1997), or to the slower development of tolerance to the anticonvulsant effect relative to other benzodiazepines, such as diazepam or clobazam (Gent et al., 1985; Rosenberg et al., 1989). The limited distribution of $\alpha 5$ subunit-containing $GABA_A$ receptors, as well as their ability to differentiate amongst benzodiazepine receptor agonists and antagonists, suggests a useful avenue for development of more selective therapeutic agents that may be useful for treating epilepsy, memory deficits, or other neurological disorders.

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