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Pharmacologic Actions of Subtype-Selective and Novel GABAergic Ligands in Rat Lines With Differential Sensitivity to Ethanol

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WONG, G., M. SARVIHARJU, M. TOROPAINEN, D. MATECKA AND E. R. KORPI. *Pharmacologic actions of subtype-selective and novel GABAergic ligands in rat lines with differential sensitivity to ethanol.* PHARMACOL BIOCHEM BEHAV 53(3) 723-730, 1996. – Alcohol-nontolerant (ANT) rats, produced by selective breeding for high sensitivity to motor-impairing effects of ethanol, have a point mutation in the cerebellar γ-aminobutyric acid type A ($GABA_A$) receptor α6 subunit, which has been proposed to underlie enhanced sensitivity to benzodiazepine agonists as well. We compared ANT and alcohol-tolerant (AT) rats using behavioral and neurochemical methods to assess the significance of α6- and nonα6-containing $GABA_A$ receptor subtypes. Motor performance in a tilting plane test was largely unaffected by a type I benzodiazepine receptor-preferring agonist, zolpidem [1-10 mg/kg, intraperitoneally (IP)], partial benzodiazepine agonists bretazenil and ZG-63 (both at 40 mg/kg, IP), and a novel broad-spectrum anticonvulsant loreclezole (40 mg/kg, IP) in both ANT and AT rats. In contrast, diazepam (10 mg/kg, IP) impaired performance of the ANT but not AT animals. These data, supported by results from brain regional autoradiography of [^{3}H]Ro15-4513 and membrane binding of [^{3}H]ZG-63 and [^{35}S]TBPS as influenced by these ligands, strongly suggest that only ligands with full agonist actions on mutant (ANT) but not wild-type (AT) α6-containing $GABA_A$ receptors are able to produce motor impairment in the ANT rats.

Alcohol sensitivity Benzodiazepine receptors $GABA_A$ receptor subtypes Zolpidem Bretazenil ZG-63 Loreclezole Selected rat lines

ALCOHOL-SENSITIVE alcohol-nontolerant (ANT) and alcohol-insensitive, alcohol-tolerant (AT) rat lines have been selectively bred for high and low sensitivity to alcohol-induced motor impairment in a tilting plane test to create an animal model for acute alcohol intoxication (5). The ANT rats are also more sensitive than AT rats (and mixed rats representing the original foundation stock for ANT and AT rats) to the motor-impairing effects of a barbiturate, sodium barbital, and a benzodiazepine receptor agonist, lorazepam (9). This strongly suggests the involvement of heterooligomeric γ-aminobutyric acid type A ($GABA_A$) receptors in this genetic alteration of drug sensitivity (10).

$GABA_A$ receptors are pentameric assemblies of several subunit proteins belonging to subunit families α, β, γ, and δ (16,23,36). Different subunit combinations form the structural basis for pharmacologic $GABA_A$-receptor subtypes (19). Ligand binding studies on brain membranes from ANT and AT rats have thus far indicated only minor differences in the numbers and affinities of, and in the coupling between, GABA and other sites in the $GABA_A$-receptor complex in brain regions other than cerebellum (20,31). A major abnormality in the $GABA_A$ receptors of ANT rats was observed in the cerebellar granule cell-specific receptor subtype (30), which contains the α6 subunit that makes the receptors normally insensitive to benzodiazepine full agonists (K_i for diazepam ~100 μM) (17,21,29). In ANT rats, the α6 containing

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"diazepam-insensitive" receptors is abnormally sensitive to benzodiazepine agonists (K_I for diazepam ~1 μM) (13,30); this has been demonstrated to be due to a single point mutation in a pharmacologically critical domain of the α6 subunit (11). In the present study, we attempted at test the hypothesis that the α6-subunit alteration accounts for the enhanced sensitivity of the ANT rats to drugs acting on $GABA_A$/benzodiazepine receptors. We compared the rat lines in their sensitivity to the motor-impairing effects of novel ligands with various binding profiles and sites in the $GABA_A$ receptors.

We tested a novel potent hypnotic, zolpidem, an imidazopyridine with preferential affinity for type I benzodiazepine receptors (K_D ~ 7 nM) (2,3,14), but without affinity (K_I > 10,000 nM) to α6 subunit-containing recombinant $GABA_A$ receptors, even when a mutation renders them sensitive to 1,4-benzodiazepine agonists (34). We first determined by ligand autoradiography whether zolpidem has affinity to ANT rat cerebellar granule cell benzodiazepine-binding sites labelled by [^{3}H]Ro15-4513, a ligand recognizing all known central benzodiazepine receptors (15,28), and then compared it with diazepam in its behavioral potency on ANT and AT rats.

To assess differences in sensitivities to partial agonists, we evaluated bretazenil, with no known preference for $GABA_A$-receptor subtypes (22,25). This ligand binds with high affinity to "diazepam-insensitive" [^{3}H]Ro15-4513 sites in cerebellar membranes from both ANT and AT rats (13). Thus, we hypothesized that it should not produce differential motor impairment between the ANT and AT rats. ZG-63, related to bretazenil in structure and pharmacologic profile (an imidazobenzodiazepine partial agonist) (7,8), was included in the evaluation because of its preference for diazepam-insensitive over diazepam-sensitive $GABA_A$/benzodiazepine receptors (37). This difference is also observable in the α6 subunit-containing receptors between the ANT and AT rat lines (39). Therefore, this ligand might have different actions on motor performance in the ANT and AT rats, although its intrinsic activity in α6 subunit-containing receptors has not been established.

Finally, we were interested in a novel nonbenzodiazepine anticonvulsant, loreclezole, which is unsedative and has a unique interaction with $GABA_A$-receptor subunits (1,32,35). It has been proposed to be active only on receptors containing β subunit(s) other than β1 (35), which renders it suitable to test the possible involvement of subunits in the differential motor impairment of the rat lines and in the cerebellar $GABA_A$-receptor function studied using modulation of ionophore-binding sites labelled by [^{35}S]TBPS [*t*-butylbicyclophosphoro[^{35}S]thionate].

METHOD

Animals

Adult male rats of the alcohol-sensitive ANT and alcohol-insensitive AT lines were maintained in groups of four to six under a 12 L : 12 D cycle (lights on at 0600 h) at an ambient temperature of 20–22°C and a relative humidity of 50 ± 5%. The rats had free access to RM1 (E) SQC pellet feed (SDS Ltd., Witham, Essex, UK) and tapwater.

Rats from the generation F_{45} were raised and maintained in an old animal facility, and rats from the generations F_{48} and F_{49} were from a new facility; the F_{48} generation was transferred there pathogen-free via hysterectomy carried out at the Central Animal Laboratory of the University of Turku, as described in detail by Sarviharju and Jaakkola (27). The hysterectomy was successful in eliminating *Mycoplasma pulmonis* and Sendai, Kilham, and Toolan H-1 viruses from the rat lines. Table 1 indicates that the difference in alcohol sensitivity between the AT and ANT rats was unaltered after the hysterectomy; however, a comparison of behavioral determinations between the two generations was slightly hampered by a significant ($p < 0.001$) difference between experiments in blood alcohol concentration, which could account for the greater impairment of the AT rats before the hysterectomy. No such difference was observed in behavioral scores for the ANT rats, suggesting that their performance was already maximally impaired at lower alcohol concentrations.

All animal experiments were approved by the Institutional Animal Care and Use Committee at Alko Group Ltd.

Materials

[^{3}H]Ro15-4513 and [^{35}S]TBPS were purchased from Du Pont de Nemours (NEN Division, Dreieich, Germany). [^{3}H]ZG-63 and unlabelled ZG-63 [(*tert*-butyl-8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo(1,5-a)(1,4)benzodiazepine-3-carboxylate] were synthesized and purified as previously described (6,7). GABA and picrotoxinin were purchased from Sigma Chemical Co. (St. Louis, MO). Flumazenil (Ro15-1788) and bretazenil (Ro16-6028) were kindly donated by F. Hoffmann-La Roche (Basle, Switzerland), diazepam by Orion Pharmaceutica (Espoo, Finland), zolpidem by Synthelabo Recherche (Bagneux, France), and loreclezole (R72063) by Janssen Pharmaceutica (Beerse, Belgium). For behavioral experiments, all drugs were suspended in 5% cum arabicum in distilled water. For neurochemical experiments, drugs were dissolved in dimethylsulfoxide. The solvent concentration in assays was <12 mM, which had no effect on [^{3}H]Ro15-4513 and [^{35}S]TBPS binding.

Tilting Plane Test (TPT)

We carried out a TPT as described by Hellevuo et al. (9), to determine the sensitivity of the animals to motor impairment after intraperitoneal (IP) administration of various drugs. In the TPT, the animal was placed on a wire cloth-covered plane, which was tilted at a constant speed. The tilting was automatically stopped by the rat's sliding backward to the lower edge of the plane, and the sliding angle was recorded. Each rat was given a three-trial predrug test, then injected with a drug and tested again 30 and 60 min later. The differ-

TABLE 1

ALCOHOL-INDUCED MOTOR IMPAIRMENT IN ANT AND AT RATS BEFORE AND AFTER HYSTERECTOMY

		F_{46} (Before Hysterectomy)			F_{49} (After Hysterectomy)		
	Sex	*n*	Impairment	BAC (mM)	*n*	Impairment	BAC (mM)
ANT	M	38	22 ± 1*	48 ± 1	29	24 ± 2*	40 ± 1
AT	M	43	9 ± 1	50 ± 1	23	3 ± 1	41 ± 1
ANT	F	40	25 ± 1*	47 ± 1	30	24 ± 2*	40 ± 1
AT	F	45	7 ± 1	49 ± 1	26	2 ± 1	40 ± 1

Alcohol-induced motor impairment was determined 30 min after an IP injection of 2 g/kg ethanol in saline, and is given as the mean change in the sliding angle (±SE, in degrees) between pretest and test under alcohol. Blood alcohol concentration (BAC) was determined from a tail-tip whole-blood sample immediately after the test under alcohol (4).

*$p < 0.001$ for the significance of the difference from the corresponding AT values.

ence in predrug and drug sliding angles was then calculated and used as the measure of motor performance under drugs. One group of ANT and AT rats (generation F_{45}) was tested first with various IP doses (0 = vehicle, 1, 3, and 10 mg/kg) of zolpidem, and then 8 days later with diazepam. Three other groups of ANT and AT rats (generation F_{48}) were tested with bretazenil, loreclezole, and ZG-63 using a single high dose (40 mg/kg, IP) to detect motor impairment, and a fourth group with diazepam (10 mg/kg, IP) to serve as a positive control for the rat line sensitivity difference.

Tissue Preparation

Drug-naive animals (generations F_{48} and F_{49}) were decapitated and the cerebral cortex and cerebellum were quickly removed, rinsed in saline, frozen on dry ice, and stored at −80°C for at least 24 h. For autoradiography, whole brains were carefully dissected and frozen on dry ice. Membranes were prepared by homogenization in 50 vol. of ice-cold 50 mM Tris-citrate buffer, pH 7.4, with a Polytron PT 10/35 (Kinematica, Kriens, Switzerland) (setting 6.5), and centrifuged at 20,000 × *g* for 20 min. The pellets were washed four times by resuspension and centrifugation. The final suspensions were stored at −80°C until use.

Ligand Binding to AT and ANT Brain Membranes

[^{3}H]ZG-63 binding assays were performed in a final volume of 500 μl consisting of 100 μl tissue (~100 μg protein), 50 μl [^{3}H]ZG-63 (2 nM), 50 μl GABA (1 μM–10 mM) and Tris-citrate buffer to volume. Binding to "diazepam-insensitive" benzodiazepine receptors was defined in the presence of 1 μM diazepam, and nonspecific binding in the presence of 10 μM flumazenil. Incubations were performed for 60 min at 0–4°C in the dark. [^{35}S]TBPS binding assays were performed with tissue (~100–200 μg protein), and 2 nM [^{35}S]TBPS, various concentrations of loreclezole with and without 5 μM GABA in 50 mM Tris-citrate buffer supplemented with 200 mM NaCl. Nonspecific binding was defined in the presence of 20 μM picrotoxinin. Incubations were performed for 90 min at 22°C.

Binding assays were terminated by filtration on Whatman GF/B glass fibre filters (Maidstone, Kent, UK) in a Brandel M-48R filtering manifold (Brandel Biochemical Research & Development Lab, Gaithersburg, MD). The filters were washed twice with 5 ml ice-cold buffer, dried, and immersed in 4 ml of Optiphase HiSafe II scintillation cocktail (Wallac, Turku, Finalnd). The radioactivities were determined with a 1410 liquid scintillation counter (Wallac) using external standardization.

Autoradiography

Autoradiographic analysis of [^{3}H]Ro15-4513 binding was carried out in 14-μm horizontal rat brain sections. The sections were cut with cryostat, thaw-mounted on gelatin-coated object glasses, dried at room temperature for 2 h, and stored dessicated at −80°C until the incubations. The sections were first preincubated in plastic slide mailers for 15 min at 0°C in 50 mM Tris-HCl (pH 7.4) buffer supplemented with 120 mM NaCl. Then the sections were incubated for 1 h in the same conditions with 5 nM [^{3}H]Ro15-4513 and various concentrations of competing ligands. This was followed by two 30-s washes in ice-cold incubation buffer, a brief dip into distilled water, and drying by air flow. The sections were exposed to Hyperfilm-[^{3}H] (Amersham, Buckinghamshire, UK) for 3–6 weeks. Then, 10 μM flumazenil reduced the signal to background level (not shown). Regional labelling intensities were quantitated from the films by using MCID M4 image analysis devices and programs (Imaging Research, St. Catharines, Canada). Binding densities for each brain area were averaged from bilateral measurements from one section. Plastic [^{3}H]-standards (Amersham) exposed simultaneously with the brain sections were used as reference with the resulting binding values given as radioactivity levels estimated for gray-matter areas (nanocuries per milligram). Sample autoradiographs were photographed as positive images to illustrate the regional effects of diazepam and zolpidem in the ANT and AT rats.

Data Analysis

Ligand-binding data were analyzed by iterative curve fitting (Inplot 4.0 and Prism; GraphPad Software, San Diego, CA). We used Student's *t*-test to assess the statistical significance of the differences between rat lines or between treatment groups within the lines (Instat; GraphPad).

RESULTS

Autoradiographic localization of [^{3}H]Ro15-4513 (5 nM) binding in the presence and absence of diazepam and zolpidem was determined in AT and ANT brain horizontal sections to compare competing ligands' affinity to the receptors in ANT cerebellar granule cell layer, and to ascertain whether the subtype-selective agonist zolpidem differently affects the binding in forebrain regions between the rat lines. Zolpidem only partially displaced granule cell layer binding in ANT rats, compared with diazepam, which caused complete displacement (Fig. 1). Similar levels of binding were detected after zolpidem and diazepam displacement in the AT sections (Fig. 1). Of the regions quantitated, the only significant rat line difference produced by zolpidem (100 μM) was observed in the cerebellar granule layer, but this effect was minor compared with that of diazepam (100 μM) (Table 2). Other regions measured, including the cerebellar molecular layer, hippocampus, and cerebral cortex, displayed no significant differences between the rat lines in the presence of zolpidem or diazepam. Basal [^{3}H]Ro15-4513 binding was greater in the granule cell layer of ANT than AT rats, consistent with its higher affinity to mutant than wild-type α6-containing recombinant receptors (Table 2) (11). A minor line difference was observed in the basal binding of hippocampus.

Based on these results, our hypothesis was that zolpidem would not produce motor impairment even in the ANT rats, compared with diazepam, because it did not potently affect the mutant α6 subunit-containing cerebellar receptors. Diazepam (10 mg/kg, IP) significantly ($p < 0.05$) impaired motor performance in the TPT in the ANT but not AT rat lines (Fig. 2). Instead, a slight improvement of performance was observed with a 3-mg/kg dose of diazepam in the AT rats. Administration of zolpidem (1, 3, and 10 mg/kg, IP) did not significantly impair motor performance in either rat line, although all animals appeared to be heavily sedated. Essentially identical results were obtained at 30 and 60 min following drug administration.

The effects of high doses of bretazenil, loreclezole, and ZG-63 were also evaluated in the TPT (Table 3). Bretazenil (40 mg/kg, IP) had no significant effect on either rat line. Loreclezole (40 mg/kg, IP) produced a small but significant ($p < 0.05$) impairment at 60 min in the ANT rats, but the performances between the lines did not differ. A significant improvement ($p < 0.05$) in motor performance was observed with ZG-63 (40 mg/kg, IP) after 30 min in ANT rats, but

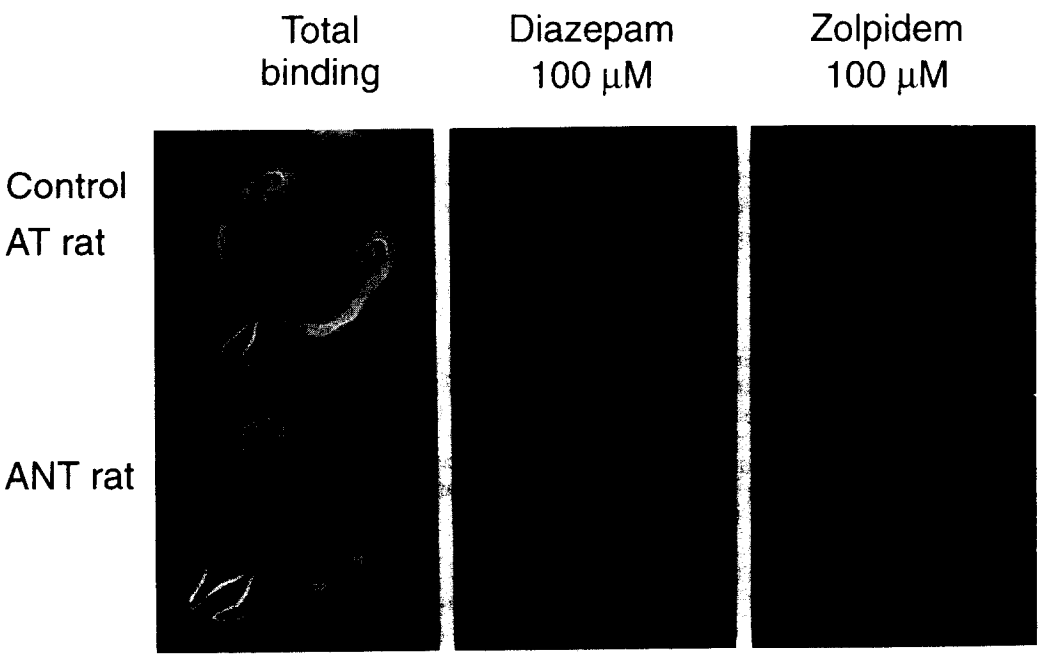

FIG. 1. Autoradiographic distribution of [^{3}H]Ro15-4513 binding sites in representative horizontal sections of ANT and AT rat brains as displaced by diazepam or zolpidem. Sections were incubated with 5 nM [^{3}H]Ro15-4513 in the presence of diazepam (100 μM) or zolpidem (100 μM) as indicated. Nonspecific binding determined in the presence of 10 μM flumazenil was at a background level (not shown).

TABLE 2

REGIONAL EFFECTS OF DIAZEPAM AND ZOLPIDEM ON THE BINDING OF [^{3}H]Ro15-4513 IN ANT AND AT RAT BRAIN SECTIONS

Rat Line	Basal (nCi/mg)	Diazepam (1 μM)	Diazepam (100 μM)	Zolpidem (3 μM)	Zolpidem (100 μM)
Cerebellar granule cell layer					
ANT	19.8 ± 1.9‡	61.4 ± 2.0†	4.0 ± 1.7‡	72.3 ± 4.8	50.6 ± 3.0‡
AT	11.0 ± 2.2	76.6 ± 11.0	51.7 ± 15.0	76.7 ± 5.5	72.0 ± 7.5
Cerebellar molecular layer					
ANT	12.6 ± 1.0	9.4 ± 3.4	2.4 ± 3.6	14.9 ± 4.3	7.0 ± 3.6
AT	12.1 ± 1.7	8.4 ± 2.6	5.0 ± 3.0	13.9 ± 3.1	6.0 ± 2.5
Entorhinal cortex					
ANT	14.5 ± 0.8	9.0 ± 2.6	2.4 ± 3.7	39.6 ± 3.4	24.0 ± 3.3
AT	14.6 ± 1.4	8.4 ± 1.8	1.4 ± 1.8	42.5 ± 1.8	27.8 ± 2.4
Hippocampus					
ANT	12.0 ± 0.7*	9.5 ± 2.7	2.6 ± 3.7	51.6 ± 4.4	30.0 ± 4.2
AT	11.0 ± 0.7	10.4 ± 2.7	1.8 ± 2.5	53.7 ± 4.1	33.8 ± 3.4
Cerebral cortex					
ANT	13.9 ± 1.0	8.0 ± 2.5	4.0 ± 2.2	30.8 ± 3.5	14.8 ± 4.1
AT	15.6 ± 2.0	7.5 ± 2.9	2.4 ± 1.3	30.0 ± 4.3	13.9 ± 3.4
Caudate nucleus					
ANT	5.3 ± 0.1	13.9 ± 5.1	8.1 ± 7.7	45.3 ± 9.4	22.7 ± 7.3
AT	5.3 ± 0.5	14.2 ± 6.4	5.4 ± 5.4	47.2 ± 6.2	22.3 ± 3.0
Olfactory bulb					
ANT	12.2 ± 1.2	9.8 ± 3.1	4.8 ± 2.6	41.4 ± 4.4	21.7 ± 3.3
AT	13.5 ± 1.8	8.3 ± 1.6	2.8 ± 2.3	41.2 ± 4.2	18.5 ± 2.8

Values are means ± SD (n = 6). Values in the presence of drugs are given as a percentage of the basal binding.

*$p < 0.05$, †$p < 0.01$, ‡$p < 0.001$ for the significance of the difference from the corresponding AT values.

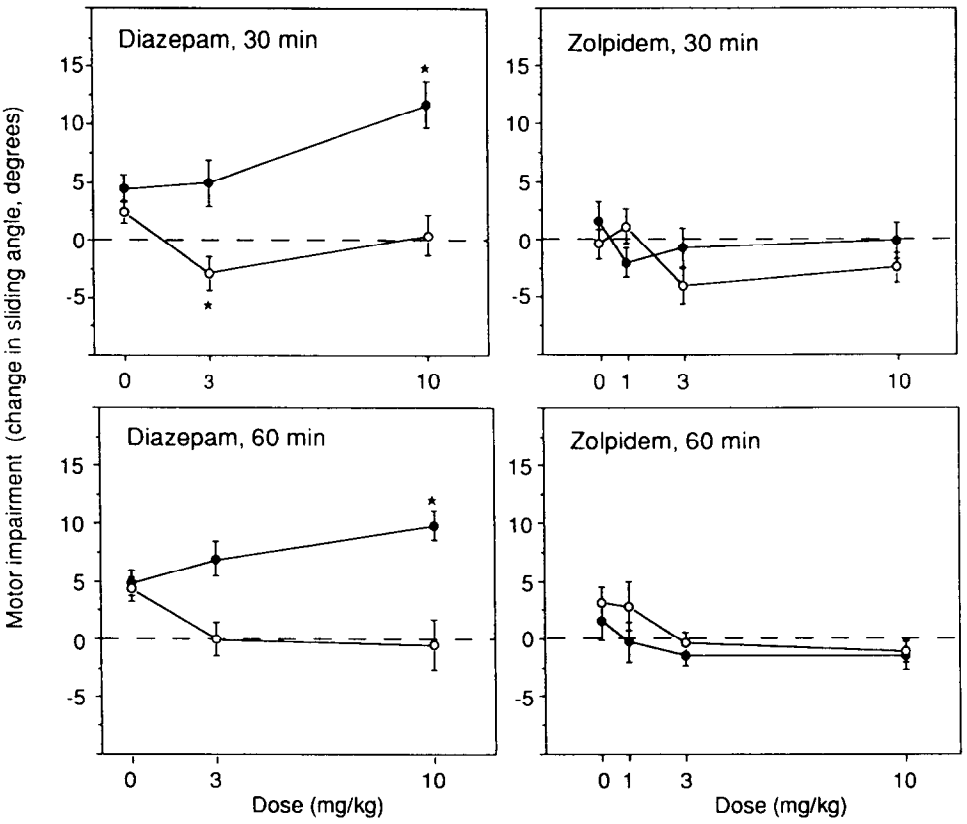

FIG. 2. Motor-impairing effects of diazepam and zolpidem in the tilting plane test. Test were performed 30 or 60 min after drug injections to ANT (●) and AT (○) rats. Changes in sliding angle are presented as degrees (value pretest − value following drug administration). The points represent the mean ± SE (n = 10–11). *$p < 0.05$ vs. vehicle-treated rats.

without a rat line difference. Improvement was not seen with the other ligands. In the control experiment with diazepam (10 mg/kg, IP), the ANT rats were significantly impaired at 30 and 60 min, with the difference from the AT rats reaching significance ($p < 0.05$) at 60 min.

Loreclezole decreased the binding of [^{35}S]TBPS in cerebellar membranes in the nominal absence of GABA but only at the highest concentration tested (Fig. 3). No apparent differences were observed between AT and ANT membranes. GABA (5 μM) decreased [^{35}S]TBPS binding, which was further enhanced by loreclezole similarly in the AT and ANT membranes.

Radioligand binding studies with [^{3}H]ZG-63 indicated no significant differences in the potency (EC_{50} = 0.6 ± 0.5 μM vs. 1.0 ± 0.8 μM, mean ± SEM, n = 3) or efficacy (E_{max} = 25 ± 5.1% vs. 20 ± 1.2%) of GABA to increase [^{3}H]ZG-63

TABLE 3

EFFECTS OF BRETAZENIL, ZG-63, AND LORECLEZOLE ON THE TILTING PLANE TEST PERFORMANCE OF ANT AND AT RATS

	Change in the sliding angle (min)			
	30		60	
Drug/Dose	ANT	AT	ANT	AT
Bretazenil/40 mg per kg	3.4 ± 6.1	−1.2 ± 5.1	1.6 ± 6.0	0.2 ± 5.2
Loreclezole/40 mg per kg	0.6 ± 2.5	−1.2 ± 3.7	2.4 ± 2.5*	0.6 ± 5.4
ZG-63/40 mg per kg	−2.6 ± 2.4*	−1.1 ± 5.8	−1.4 ± 2.8	0.2 ± 6.1
Diazepam/10 mg per kg	3.7 ± 4.0*	−0.2 ± 5.0	7.2 ± 6.8*†	1.6 ± 3.8

Values are (Pretest − Drug Test Sliding Angles), in degrees ±SD for nine ANT and 10 AT rats.

*$p < 0.05$ for the significance of the change in sliding angle.

†$p < 0.05$ for the significance of the difference between the rat lines.

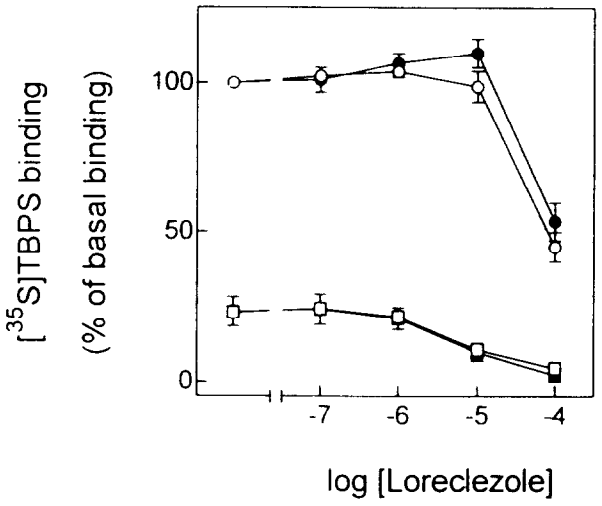

FIG. 3. Effects of loreclezole on picrotoxinin-sensitive [^{35}S]TBPS binding in ANT (closed symbols) and AT (open symbols) cerebellar membranes. [^{35}S]TBPS binding was determined in the absence (circles) or presence (squares) of 5 μM GABA. The results are expressed as percentages (mean ± SE for three determinations) of basal binding, determined in the absence of added GABA (100%). Values to the left of the gap are in the absence of loreclezole.

binding in AT and ANT cortical membranes, respectively (Fig. 4A). [^{3}H]ZG-63 binding to cerebellar "diazepam-insensitive" benzodiazepine receptors (in the presence of 1 μM diazepam) was decreased by GABA more potently (EC_{50} = 1.5 ± 0.1 μM vs. 16.4 ± 6.3 μM; $p < 0.05$) in ANT than AT, without signficant differences in efficacy (E_{max} = −24 ± 1.9% vs. −15 ± 4.8%) (Fig. 4B).

DISCUSSION

The results clearly demonstrate that partial agonists and the subtype-selective ligand zolpidem are not able to produce impairment of motor function even in the ANT rats. The present results extend the enhanced lorazepam sensitivity of the ANT rats (9) to diazepam, another benzodiazepine agonist. These results are consistent with the involvement of a mutation of cerebellar granule cell-specific $GABA_A$ α6-receptor subunit in ANT rats, because the only benzodiazepine receptor ligands able to produce enhanced motor impairment in this rat line are the full agonists, which have increased affinity to the mutant cerebellar receptors. The present results also suggest that other benzodiazepine full agonists, such as triazolam and midazolam, which are insensitive at the diazepam-insensitive site (38), would produce impaired motor performance in the ANT but not AT rats, although this remains to be tested.

Zolpidem, tested up to a dose of 10 mg/kg, which produces a maximal sedative effect on the rat brain (24), had no effect on motor impairment, although it was clearly sedative in both ANT and AT rats (unpublished observation). Once awakened, they exhibited normal postural adjustment to the tilting of the plane. Zolpidem displaced [^{3}H]Ro15-4513 binding in the cerebellar granule cell layer only slightly more in ANT than AT rats. Both of these actions were observed with diazepam, implicating the $GABA_A$ α6-receptor mutant subunit in the neurochemical and behavioral differences between the two compounds and the two rat lines.

These results are also consistent with a previous report demonstrating zolpidem to be three times more potent in suppressing operant responding than in impairing motor performance (rotarod), whereas nonselective triazolam and midazolam are two to four times more active in the rotarod test (40). The preferential binding of zolpidem to type I benzodiazepine receptors (K_I = 19 nM) (26) strongly suggests that this subtype, presumably constituted from α1βxγ2 subunits (18), functions similarly in ANT and AT lines. Autoradiography (Table 2, Fig. 1) further highlighted the similarity of type I $GABA_A$ receptors between ANT and AT rats, as zolpidem displaced similar proportions of [^{3}H]Ro15-4513 binding in both rat lines in many forebrain regions. The autoradiographs displayed the nearly complete (>90%) displacement of [^{3}H]Ro15-4513 binding outside the cerebellum by diazepam but not zolpidem. This took place similarly in both rat lines, which can be interpreted to mean that the zolpidem-insensitive (α5βxγ2/3 and α1/2/3 βxγ3, K_I > 10,000 nM) and zolpidem-sensitive (α2/3βxγ2, K_I ~ 400 nM) portions of type II binding (18,26) are also similar in ANT and AT lines. The significant difference observed in the [^{3}H]Ro15-4513 binding at 100

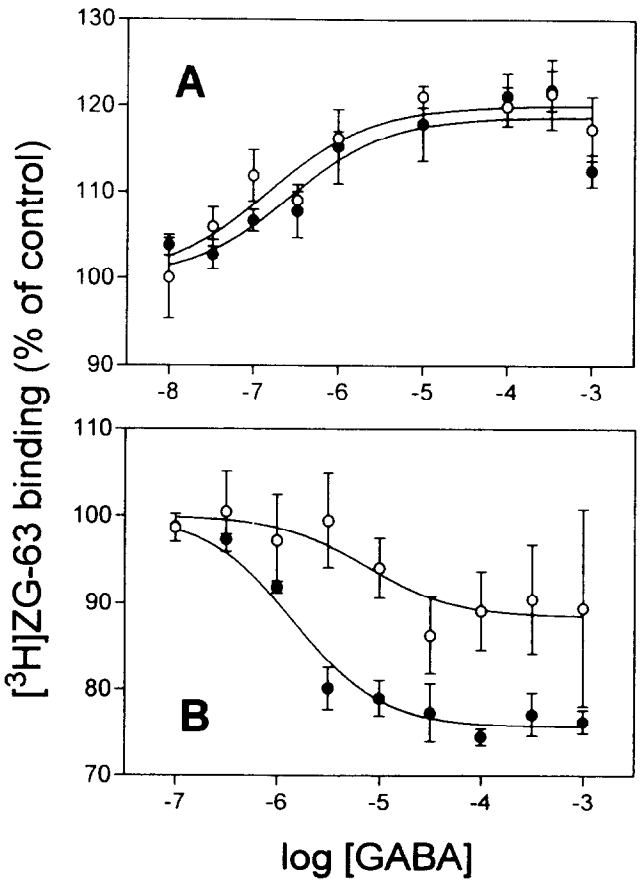

FIG. 4. Effects of GABA on flumazenil-sensitive [^{3}H]ZG-63 binding in cortical (A) and cerebellar (B) membranes from ANT (●) and AT (○) rats. [^{3}H]ZG-63 binding was determined in the presence of various GABA concentrations. The cerebellar membranes were incubated with 1 μM diazepam to determine the diazepam-insensitive component. The results are expressed as percentages (mean ± SE of three determinations) of control binding, determined in the absence of added GABA (100%).

μM zolpidem between the rat lines in the cerebellar granule cell layer (Table 2) is likely due to an affinity difference between wild-type and mutant α6-containing GABA$_A$ receptors. It is unlikely a result of changes in relative proportions of zolpidem-insensitive receptors other than α6-containing ones, as they are not appreciably expressed in the cerebellum (2,3).

Bretazenil binds with high affinity (K_i 3–15 nM) to cerebellar diazepam-insensitive wild-type and mutant α6-containing GABA$_A$ receptors in ANT, AT, and Wistar rats (13,38). It did not impair TPT motor performance, possibly because of its weak efficacy as an agonist (22,25).

Another partial agonist, ZG-63 (37), binds to cerebellar diazepam-insensitive ANT receptors with high affinity and approximately sevenfold selectivity over AT (K_i 1 and 7 nM, respectively) (39). This compound has three- to fourfold selectivity for diazepam-insensitive compared with other diazepam-sensitive GABA$_A$ receptors (6). Similar upmodulation of [^{3}H]ZG-63 binding by GABA in cerebrocortical membranes of the ANT and AT rats (Fig. 3) is consistent with its agonistic profile and a lack of difference in GABA$_A$ receptors in this brain region between the rat lines. Our data on cerebellar [^{3}H]ZG-63 binding indicate a difference between the rat lines, because GABA apparently enhanced the displacement of [^{3}H]ZG-63 binding by diazepam more from the ANT than AT membranes.

In the present study, ZG-63 was the only compound tested that elicited a significant though small improvement in motor performance of the ANT rats. Although improvement in the TPT performance can also result from a mild anxiolytic effect, as illustrated in Fig. 2 for a lower dose of diazepam in the AT rats, it is tempting to speculate that the ~sevenfold preference of ZG-63 for cerebellar receptors in ANT rather than AT rats (39) may be linked to its motor performance-enhancing properties, in contrast to bretazenil (13). This would suggest that ZG-63 and bretazenil interact with diazepam-insensitive GABA$_A$ receptors in a different manner, with ZG-63 possibly acting as an inverse agonist. Further studies are warranted to establish the intrinsic profiles of these ligands in mutant and wild-type α6-containing receptors and the role of GABA in their actions. A recent study by Harris et al. (8) demonstrated that ZG-63 could increase alcohol-induced sleep time in mice, which could be potentiated by preferential blockade of diazepam-sensitive sites by ZK 93426, supporting the idea that several GABA$_A$-receptor subtypes are involved in the behavioral effects of ZG-63.

Interactions of loreclezole with ANT and AT rat lines demonstrate similarities in the unique GABA$_A$ receptor-binding site for this ligand. A recent report demonstrated that a single amino acid on the β2 and β3 subunits is critical for the action of loreclezole on GABA$_A$ receptors (35). The lack of rat line differences in either motor impairment elicited by loreclezole at an ED$_{50}$ dose for ataxia in rats (33) or [^{35}S]TBPS binding response in the presence of loreclezole strongly suggests that the loreclezole-binding site has not been altered and is not responsible for increased GABAergic sensitivity in the ANT rat line. Although it is difficult to dissociate the effects on α6- and nonα6-containing receptors with this ligand, the higher GABA sensitivity of α6-containing receptors (12) suggests that both components are involved in the absence of exogenous GABA, and only a nonα6 component in the presence of 5 μM GABA.

Taken together, these data demonstrate that not all benzodiazepine receptor agonists are able to produce enhanced motor impairment in ANT rats, which implies that the compounds producing motor impairment in the TPT need to be very efficacious or act only on certain receptor subtypes. Thus, it can be suggested that binding to cerebellar α6-containing GABA$_A$ receptors is necessary, but not sufficient, to impair motor performance of mutant ANT rats, and that the binding must be coupled to efficient agonistic action on this receptor population.

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