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The cerebellar $GABA_AR$ α6-R100Q polymorphism alters ligand binding in outbred Sprague–Dawley rats in a similar manner as in selectively bred AT and ANT rats

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Abstract

The alcohol-tolerant AT and alcohol-nontolerant ANT rat lines have been selectively bred for innate sensitivity to ethanol-induced motor impairment. The cerebellar $GABA_A$ receptor ($GABA_AR$) α6 subunit alleles α6-100R and α6-100Q are segregated in the AT and ANT rats, respectively. This α6 polymorphism might explain various differences in pharmacological properties and density of $GABA_ARs$ between the rat lines. In the present study, we have used nonselected outbred Sprague–Dawley rats homozygous for the α6-100RR (RR) and α6-100QQ (QQ) genotypes to show that these RR and QQ rats display similar differences between genotypes as AT and ANT rat lines. The genotypes differed in their affinity for [^{3}H]Ro 15-4513 and classic benzodiazepines (BZs) to cerebellar "diazepam-insensitive" (DZ-IS) binding sites, in density of cerebellar [^{3}H]muscimol binding and in the antagonizing effect of furosemide on GABA-induced inhibition of [^{3}H]EBOB binding. The results suggest the involvement of α6-R100Q polymorphism in these line differences and in the differences previously found between AT and ANT rats. In addition, the α6-R100Q polymorphism induces striking differences in [^{3}H]Ro 15-4513 binding kinetics to recombinant α6β3γ2s receptors and cerebellar DZ-IS sites. Association of [^{3}H]Ro 15-4513 binding was ~10-fold faster and dissociation was ~3–4-fold faster in DZ-IS α6βγ2 receptors containing the α6-100Q allele, with a resulting change of ~2.5-fold in equilibrium dissociation constant (K_D). The results indicate that in addition to the central role of the homologous α6-100R/Q (α1-101H) residue in BZ binding and efficacy, this critical BZ binding site residue has a major impact on BZ binding kinetics.

Keywords: Cerebellar granule cell; Benzodiazepine; $GABA_A$ receptors; Ethanol sensitivity; Ro 15-4513; Selected rat lines

Introduction

Selective breeding of rodent lines has been used as a tool to identify genes that mediate behavioral differences related to the selection criteria used. At the Biomedical Research Center, Alko Ltd, two rat lines, the AT and ANT rats, differing in their innate sensitivity to motor-impairing effects of ethanol have been produced (Eriksson and Rusi, 1981). The ethanol-sensitive ANT rats are also more sensitive than the ethanol-insensitive AT rats to $GABA_A$ receptor-($GABA_AR$) positive modulators lorazepam, diazepam, and barbital (Hellevuo et al., 1989; Wong et al., 1996b), suggesting the involvement of $GABA_A$ergic mechanisms mediating the sensitivity differences between the rat lines.

Various differences have been found between the AT and ANT lines in characteristics of cerebellar $GABA_ARs$. The density of high-affinity $GABA_A$ agonist ([^{3}H]muscimol) binding sites is lower in ANT than in AT rats (Malminen and Korpi, 1988; Uusi-Oukari and Korpi, 1989). The affinity of "classic" benzodiazepine (BZ) agonists to α6 subunit-containing, "diazepam-insensitive" (DZ-IS) binding sites in α6βγ2 receptors is about 100-fold higher in ANT than in AT rats (Uusi-Oukari and Korpi, 1990, 1991). This line difference is dependent on a single-point mutation in the α6 codon 100 (CGA → CAA) in ANT rats leading to an amino acid change from arginine (R) to glutamine (Q) (Korpi et al., 1993). The homologous position in α1,2,3,5 receptors is a histidine residue, which has been shown to be a critical residue for classic BZ binding at the αγ2 subunit interface (Wieland et al., 1992). The α6-R100Q mutation increases the affinity of [^{3}H]Ro 15-4513 to DZ-IS binding sites (Mäkelä et al., 1995; Uusi-Oukari and Korpi, 1990). The allosteric interaction between GABA and classic BZs in DZ-IS sites was found to be present only in ANT rats (Korpi et al., 1993; Uusi-Oukari and Korpi,

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1992). Furthermore, furosemide, a $GABA_AR$ antagonist selective for α6 subunit-containing receptors (Korpi et al., 1995) is less efficient in enhancing basal binding of the $GABA_AR$-selective channel blocker [^{35}S]TBPS and less efficient in antagonizing GABA-induced inhibition of [^{35}S] TBPS binding in ANT rats than in AT rats (Mäkelä et al., 1996, 1999). The same α6-R100Q mutation was later found to be enriched in the Sardinian non–alcohol-preferring (sNP) rat line (Saba et al., 2001), and also in rats selected for alcohol (non)preference (Carr et al., 2003), and it was shown to be a rather frequent naturally occurring α6 polymorphism in some Sprague–Dawley laboratory rat colonies (Hanchar et al., 2005).

The higher affinity and functional sensitivity of cerebellar α6βγ2 receptors in ANT than in AT rats to classic BZs suggest that the α6-100Q mutation determines the increased BZ-induced motor impairment in ANT rats (Korpi et al., 1993). However, the α6-R100Q mutation expressed in α6β2/3γ2 combination did not affect GABA or ethanol sensitivity (Hanchar et al., 2005; Korpi et al., 1993). In contrast, it was shown that α6-100Q mutation in the ethanol-sensitive receptor subtype α6β3δ (Wallner et al., 2003), while not affecting GABA sensitivity, dramatically increases ethanol sensitivity in the ethanol concentration range of 3–30 mM in recombinantly expressed receptors and in cerebellar granule cells in slices (Hanchar et al., 2005), suggesting the involvement of α6β3δ receptors in alcohol sensitivity differences between AT and ANT rats. The increased ethanol sensitivity seen with the R100Q mutation is consistent with earlier work using receptors reconstituted (by injection of cerebellar vesicles from α6-100RR and α6-100QQ rats) in oocytes (Sanna et al., 2003). Higher functional sensitivity to ethanol and BZs was also demonstrated in ANT when compared with AT cerebellar synaptoneurosomes (Schmid et al., 1999). However, using patch-clamp electrophysiological techniques in cerebellar slices of outbred Sprague–Dawley rats homozygous for the α6-100R and α6-100Q alleles, Botta et al. (2007) were not able to detect genotype-dependent ethanol sensitivity differences. In addition, crossbreeding studies using AT and ANT animals did not support the conclusion that the α6-R100Q polymorphism is important for differential ethanol sensitivity (Radcliffe et al., 2004). The controversy surrounding the ethanol sensitivity of α4/6βδ-$GABA_ARs$ and whether this is a solution for the ethanol/$GABA_AR$ dilemma is discussed in details in a special issue of the Journal Alcohol, where the issue is introduced by an editorial by Lovinger and Homanics (2007).

In the present study, we have compared cerebellar and hippocampal $GABA_ARs$ of Sprague–Dawley rats homozygous for the α6-100R and α6-100Q alleles. We focused on receptor differences found between AT and ANT rats to reveal if these differences are related to α6-R100Q polymorphism. In addition, we characterized the effects of the polymorphism on cerebellar [^{3}H]Ro 15-4513 binding kinetics to cerebellar DZ-IS binding sites and recombinant α6β3γ2 receptors.

Materials and methods

Animals

Sprague–Dawley rats obtained from Charles River (Hollister, CA) were genotyped as described in Hanchar et al. (2005) to identify animals homozygous for the α6-100R and α6-100Q polymorphisms. A total of 14 RR and 17 QQ adult rats were used for the studies. The rats used in experiments of [^{3}H]Ro 15-4513 binding kinetics were 6–12-month-old males, whereas rats used in other experiments were 3–4-month-old males. The animals were killed by decapitation, their brains were removed, and the cerebelli and hippocampi frozen on dry ice and stored at −70°C. All procedures were in accordance with protocols approved by the University of California at Los Angeles Chancellor's Animal Research Committee and by the Institutional Animal Care and Use Committee of the University of Turku.

Reagents

The radioligands [propyl-2,3-^{3}H]EBOB (specific activity 48 Ci/mmol), [methylene-^{3}H]muscimol (18 Ci/mmol), and [7,9-^{3}H]Ro 15-4513 (28 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA). Flumazenil (Ro 15-1788) was a gift from F. Hoffmann-La Roche Ltd (Basel, Switzerland). Diazepam, GABA, and picrotoxin were from Sigma Chemical Co. (St. Louis, MO).

Recombinant $GABA_AR$ expression in HEK 293 cells

Human embryonal kidney (HEK) 293 cells were transfected with rat cDNAs under the control of CMV promoter (α1, α6-100R, or α6-100Q:β3:γ2S, 1:1:2) as described in Meera et al. (1997) and the cells were harvested 48 h after transfection. The cells were washed with phosphate-buffered saline (PBS), homogenized in PBS using an Ultra-Turrax and stored frozen at −70°C. Before binding assays, the suspensions were thawed, washed once with ice-cold assay buffer by resuspension and centrifugation, and homogenized in ice-cold assay buffer with an Ultra-Turrax.

[^{3}H]Ro 15-4513 binding assay

Cerebellar and hippocampal membranes were prepared and [^{3}H]Ro 15-4513 binding assays performed essentially as described in Uusi-Oukari and Korpi (1990). Tris-HCl (50 mM, pH 7.4) containing 120 mM NaCl was used as the incubation buffer. Nonspecific binding was determined in the presence of 10 μM flumazenil. Triplicate samples were incubated in an ice-water bath in the dark with shaking for 1 h in a total volume of 300 μL. DZ-IS [^{3}H]Ro 15-4513 binding was determined in the presence of 100 μM diazepam to differentiate between RR and QQ binding sites. Receptors containing the α6-100Q allele display 1 μM diazepam affinity (Mäkelä et al., 1995; Uusi-Oukari and Korpi, 1992). Diazepam at 100 μM displaces essentially all binding

in QQ rats while hardly affecting α6-100R receptors (Mäkelä et al., 1995; Uusi-Oukari and Korpi, 1992). The incubation was terminated by filtration of the samples with a Brandel Cell Harvester (model 48R, Gaithesburg, MD) onto Whatman GF/B filters (Whatman International Ltd., Maidstone, UK). The samples were rinsed twice with 4–5 mL of ice-cold incubation buffer. Air-dried filters were immersed in 4 mL of Optiphase HiSafe 2 scintillation fluid (Wallac, Turku, Finland) and radioactivity determined in a Wallac model 1410 liquid scintillation counter (Wallac, Turku, Finland). Nonspecific binding was subtracted from total binding and from binding in the presence of diazepam to get total specific binding and DZ-IS binding, respectively. DZ-IS binding was subtracted from total specific binding to get diazepam-sensitive (DZ-S) binding.

Measurement of [^{3}H]Ro 15-4513 binding kinetics

To measure association of [^{3}H]Ro 15-4513 binding, cerebellar membranes of RR and QQ rats were incubated in incubation buffer with 5 nM [^{3}H]Ro 15-4513 in the absence and presence of 1 μM diazepam (DZ-IS binding) or 10 μM flumazenil (nonspecific binding) in conditions previously mentioned. Diazepam at 1 μM displaces essentially all DZ-S binding, but only 50% of DZ-IS binding in QQ membranes thus allowing measurement of DZ-IS binding selectively also in QQ cerebellum. The incubations were terminated at various time points. To measure dissociation of [^{3}H]Ro 15-4513 binding, triplicate samples of membranes were first preincubated in a total volume of 300 μL for 1 h with 5 nM [^{3}H]Ro 15-4513 in the absence and presence of 1 μM diazepam or 10 μM flumazenil. The dissociation was started by adding 100 μL of 40 μM flumazenil to the incubation mixtures to reach a final 10 μM flumazenil concentration in all tubes. The tubes were mixed and incubations terminated at various time points as previously mentioned.

[^{3}H]Muscimol binding assay

Cerebellar membranes were prepared and saturation analysis of [^{3}H]muscimol binding performed essentially as described in Uusi-Oukari and Korpi (1989). The radioligand was used at a concentration range of 0.5–20 nM. Nonspecific binding was determined in the presence of 1 mM GABA. Triplicate reaction mixtures in 50 mM Tris-citrate buffer, pH 7.4, were incubated in ice-water bath in the dark with shaking for 30 min. The final incubation volume was 300 μL. The incubation was terminated as described for [^{3}H]Ro 15-4513 binding.

[^{3}H]EBOB binding assay

Cerebellar membranes for binding of [^{3}H]EBOB, a ligand binding to the same site as the more commonly used [^{35}S]TBPS, were prepared and [^{3}H]EBOB binding assay performed essentially as described in Uusi-Oukari and Maksay (2006). Triplicate samples of cerebellar membranes were incubated at room temperature with shaking for 2 h in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl with 1 nM [^{3}H]EBOB in a total volume of 400 μL in the absence and presence of 5 μM GABA with or without 300 μM furosemide. Nonspecific binding was determined in the presence of 100 μM picrotoxin. The incubation was terminated as described for [^{3}H]Ro 15-4513 binding.

Protein measurement

In all ligand-binding studies, protein concentrations of membranes were determined with the Bio-Rad Coomassie blue dye-based protein assay kit (Hercules, CA) according to manufacturer's instructions.

Data analysis

Saturation isotherms for estimation of K_D and B_{max} values, and association and dissociation curves for estimation of association and dissociation rate constants were analyzed with Prism 5 software (Graph Pad, San Diego, CA). Two-tailed unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to assess statistical significances of the differences between the groups. *P* values of less than .05 were considered significant.

Results

[^{3}H]Ro 15-4513 binding to brain membranes and to recombinant receptors

Cerebellar DZ-IS [^{3}H]Ro 15-4513 binding was determined from all animals used in the study. The portion of 5 nM [^{3}H]Ro 15-4513 binding in the presence of 100 μM diazepam was 21.9 ± 0.9% and 1.0 ± 0.1% of total binding in membranes of RR and QQ rats, respectively. The portion of DZ-IS binding was 20–25% in all RR samples, whereas QQ samples under these conditions displayed only small amounts of DZ-IS [^{3}H]Ro 15-4513 binding. The values are consistent with previous studies on AT and ANT rat lines and confirm the $GABA_AR$ α6-100RR and α6-100QQ rat genotypes.

The association and dissociation rates of total, DZ-S, and DZ-IS [^{3}H]Ro 15-4513 binding were determined using cerebellar membranes from RR and QQ rats (Figs. 1 and 2, Table 1). Association and dissociation rates of total and DZ-S binding were very similar in RR and QQ membranes (Fig. 1, Table 1). In contrast, the K_{on} value of DZ-IS binding was 7.4-fold lower in RR than in QQ membranes indicating slower association of [^{3}H]Ro 15-4513 to DZ-IS receptors in RR membranes. The same applies to dissociation of DZ-IS [^{3}H]Ro 15-4513 binding. The K_{off} value was 2.9-fold lower in RR than in QQ membranes indicating slower [^{3}H]Ro 15-4513 dissociation in RR membranes (Fig. 2, Table 1). The calculation of K_D values (K_{off}/K_{on}) for [^{3}H]Ro

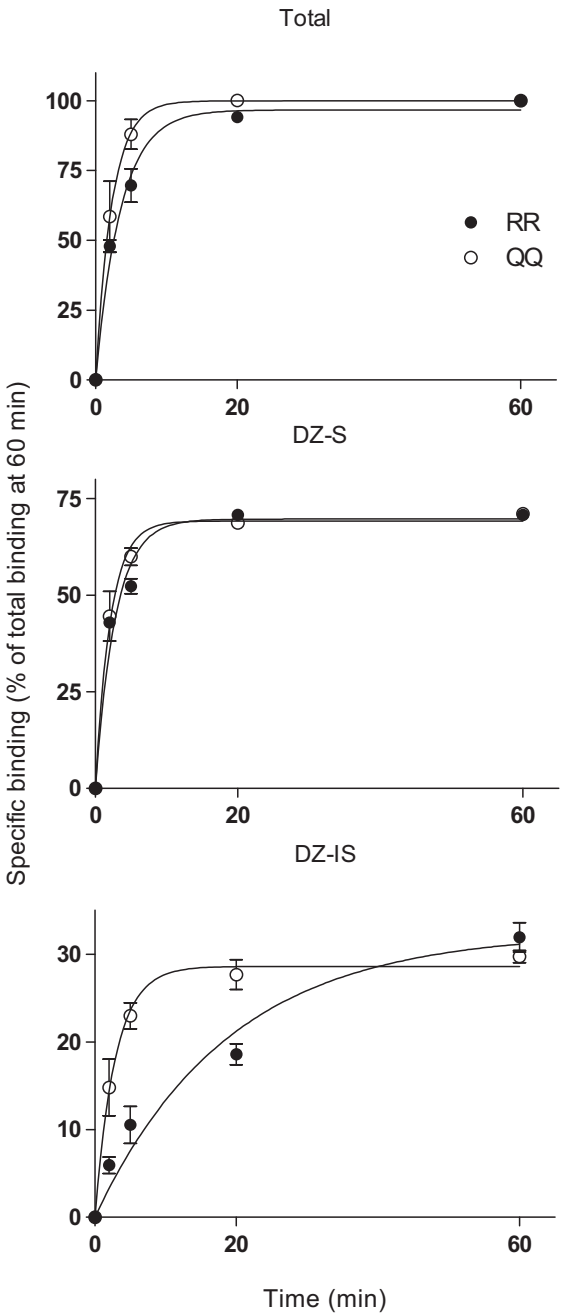

Fig. 1. Association of total, diazepam-sensitive (DZ-S), and diazepam-insensitive (DZ-IS) [^{3}H]Ro 15-4513 binding to cerebellar membranes from α6-100RR (RR) and α6-100QQ (QQ) rats (mean ± standard error of the mean of three independent experiments made in triplicate). DZ-IS binding was measured in the presence of 1 μM diazepam.

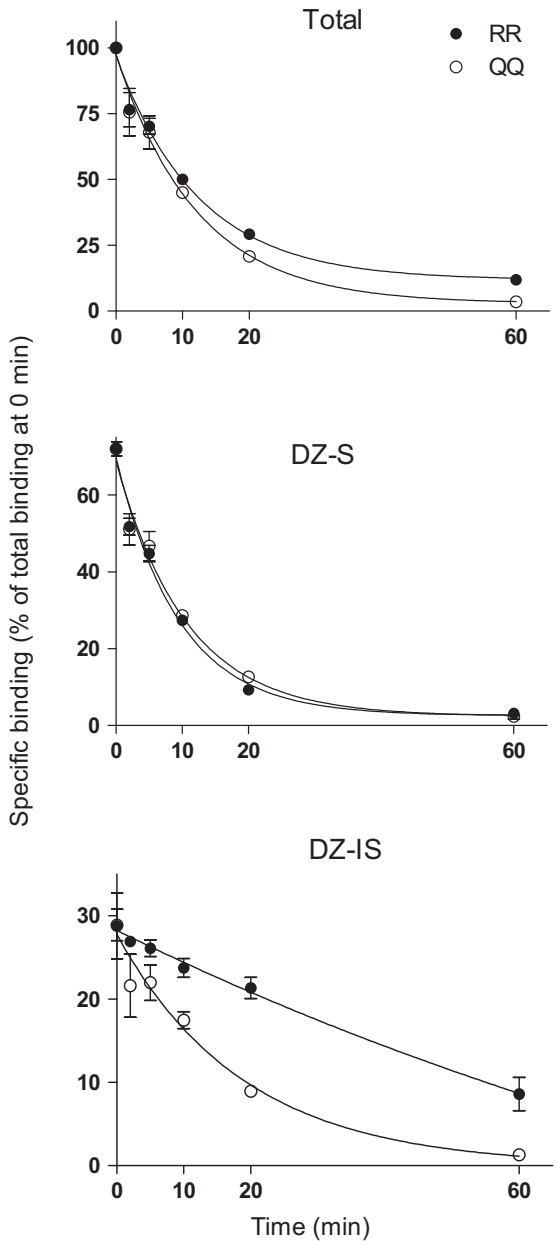

Fig. 2. Dissociation of total, diazepam-sensitive (DZ-S), and diazepam-insensitive (DZ-IS) [^{3}H]Ro 15-4513 binding from cerebellar membranes from α6-100RR (RR) and α6-100QQ (QQ) rats (mean ± standard error of the mean of three independent experiments made in triplicate). DZ-IS binding was measured in the presence of 1 μM diazepam.

15-4513 binding were 1.6 nM and 1.4 nM (DZ-S) and 2.3 nM and 0.9 nM (DZ-IS) for RR and QQ rats, respectively (Table 1).

Association rate of [^{3}H]Ro 15-4513 binding to DZ-S α1β3γ2 recombinant receptors was comparable to that of [^{3}H]Ro 15-4513 binding to DZ-S receptors in RR and QQ membranes (Table 1, Fig. 3A). Similarly, dissociation rate of the binding to α1β3γ2 receptors was indistinguishable from dissociation of DZ-S binding in cerebellar membranes (Table 1, Fig. 3B). Association and dissociation rates of [^{3}H]Ro 15-4513 binding to α6-100R-β3γ2 recombinant receptors and DZ-IS binding to cerebellar membranes of RR rats were almost identical, whereas the association rate constant was lower in DZ-IS binding to QQ rat cerebellar membranes than to α6-100Q-β3γ2 recombinant receptors (Table 1, Fig. 3A, B). This difference between QQ recombinant receptors and membranes is likely because of 1 μM diazepam present with cerebellar membranes. The K_i of diazepam to DZ-IS sites in QQ animals is 1 μM (Uusi-Oukari and Korpi, 1992)

Table 1
Association and dissociation rate constants of [^{3}H]Ro 15-4513 binding in cerebellar membranes of RR and QQ rats and in recombinant receptors expressed in HEK cells

	K_{on} ($M^{-1} \times min^{-1}$]	K_{off} (min^{-1})	K_D (K_{off}/K_{on}) (nM)
Total binding			
RR	$4.6 \pm 0.4 \times 10^7$	0.063 ± 0.002	1.4 ± 0.1
QQ	$7.6 \pm 1.1 \times 10^7$	0.080 ± 0.006	1.1 ± 0.3
DZ-S binding			
RR	$6.0 \pm 0.2 \times 10^7$	0.097 ± 0.004	1.6 ± 0.1
QQ	$6.9 \pm 0.9 \times 10^7$	0.090 ± 0.008	1.4 ± 0.3
DZ-IS binding			
RR	$8.2 \pm 0.3 \times 10^6$	0.019 ± 0.001	2.3 ± 0.1
QQ	$6.1 \pm 0.8 \times 10^{7\#\#}$	$0.055 \pm 0.001^{\#\#\#}$	$0.9 \pm 0.2^{\#\#\#}$
Recombinant receptor			
α1β3γ2	$3.4 \pm 0.2 \times 10^{7***}$	$0.098 \pm 0.010^{**}$	$2.9 \pm 0.3^{*}$
α6-100R-β3γ2	$8.0 \pm 0.8 \times 10^{6***}$	0.018 ± 0.002	$2.4 \pm 0.4^{*}$
α6-100Q-β3γ2	$8.7 \pm 0.2 \times 10^{7***}$	$0.083 \pm 0.013^{**}$	1.0 ± 0.1

RR = α6-100RR; QQ = α6-100QQ; DZ-S = diazepam-sensitive; DZ-IS = diazepam-insensitive; K_D = dissociation constant; K_{on} = association rate constant; K_{off} = dissociation rate constant; HEK = human embryonal kidney. Total, DZ-S, and DZ-IS binding to cerebellar membranes, mean ± standard error of the mean (S.E.M.) values ($n = 3$, measured in triplicate). $^{\#\#\#}P < .001$, $^{\#\#}P < .01$, significantly different from the corresponding RR value (unpaired t-test); recombinant receptors, mean ± S.E.M. ($n = 3$, measured in triplicate); $^{***}P < .001$, significantly different from two other groups; $^{**}P < .01$, significantly different from α6-100R value; $^{*}P < .05$, significantly different from α6-100Q value; one-way ANOVA followed by Tukey's post hoc test.

indicating that diazepam competes with [^{3}H]Ro 15-4513 in binding to these sites. The association rate constant of [^{3}H]Ro 15-4513 binding was 11-fold lower in α6-100R-β3γ2 than in α6-100Q-β3γ2 receptors (Table 1, Fig. 3A) ($P < .001$, two-tailed unpaired t-test). The dissociation rate constant in α6-100Q-β3γ2 receptors was 4.6-fold larger than that in α6-100R-β3γ2 receptors (Table 1, Fig. 3B) ($P < .05$, two-tailed unpaired t-test). The calculated K_D values (K_{off}/K_{on}) of [^{3}H]Ro 15-4513 binding to recombinant receptors were 2.9 nM (α1β3γ2), 2.3 nM (α6-100R-β3γ2), and 1.0 nM (α6-100Q-β3γ2) (Table 1). The association and dissociation kinetics of [^{3}H]Ro 15-4513 binding were in a similar manner slower also in α6-100R-β2γ2 recombinant receptors when compared with α6-100Q-β2γ2 receptors (L.-S. Kontturi and M. Uusi-Oukari, unpublished results) indicating that the difference in binding kinetics is independent on β variant present in the receptor.

Because association of [^{3}H]Ro 15-4513 binding to RR cerebellar membranes in the presence of diazepam (DZ-IS binding) and to α6-100R-β3γ2 recombinant receptors was very slow, we tested longer incubation periods. A 2-h incubation with 5 nM [^{3}H]Ro 15-4513 yielded 23% higher binding both to RR cerebellar membranes in the presence of diazepam and to α6-100R-β3γ2 recombinant receptors (Fig. 4).

Saturation analysis of [^{3}H]Ro 15-4513 binding to hippocampal membranes revealed 23% higher B_{max} value in RR than in QQ rats (Fig. 5). This difference, however, did not reach statistical significance ($P = .063$).

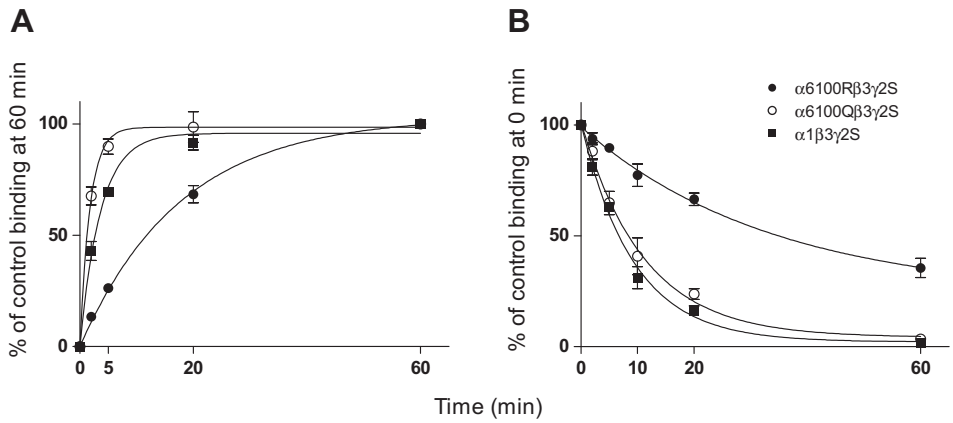

Fig. 3. A. Association kinetics of 5 nM [^{3}H]Ro 15-4513 to α1β3γ2, α6-100R-β3γ2 and α6-100Q-β3γ2 recombinant receptors expressed in HEK cells. B. Dissociation of [^{3}H]Ro 15-4513 binding from α1β3γ2, α6-100R-β3γ2 and α6-100Q-β3γ2 recombinant receptors expressed in human embryonal kidney cells (mean ± standard error of the mean, $n = 3$ independent experiments made in triplicate).

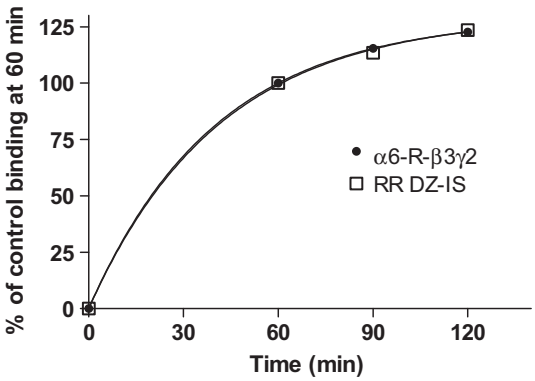

Fig. 4. Association of [^{3}H]Ro 15-4513 to α6-100R-β3γ2 recombinant receptors and to cerebellar membranes from α6-100RR (RR) rats using longer incubation times (mean values from two independent experiments made in triplicate). DZ-IS binding was measured in the presence of 1 μM diazepam.

[^{3}H]Muscimol binding to cerebellar membranes

The B_{max} value of [^{3}H]muscimol binding to cerebellar membranes was lower in QQ (1.46 ± 0.10 pmol/mg protein, mean ± standard error of the mean) than in RR (1.84 ± 0.14 pmol/mg protein) animals ($P < .05$, two-tailed unpaired *t*-test; RR, $n = 7$; QQ, $n = 8$) (Fig. 6). The K_D values of cerebellar [^{3}H]muscimol binding did not differ between the rat lines (2.21 ± 0.13 and 2.52 ± 0.22 nM for RR and QQ rats, respectively).

[^{3}H]EBOB binding to cerebellar membranes

The GABA-antagonizing effect of furosemide on [^{3}H]EBOB binding was studied in cerebellar membranes of RR and QQ rats. GABA (5 μM) inhibited basal binding of 1 nM [^{3}H]EBOB by 65–70% (Fig. 7). The effect of GABA on the binding did not significantly differ between RR and QQ rats. However, furosemide (300 μM) significantly antagonized the effect of GABA on [^{3}H]EBOB binding only in RR rats ($P < .05$, one-way ANOVA followed by Tukey's post hoc test).

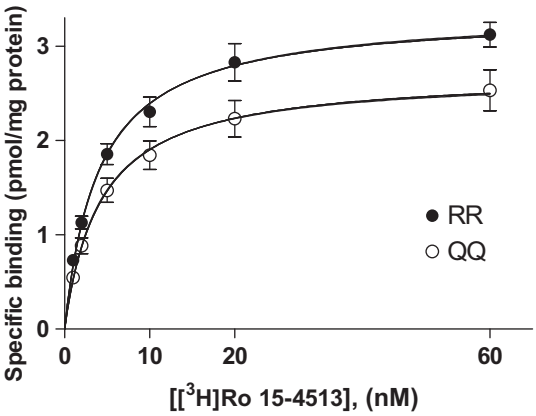

Fig. 5. Saturation analysis of [^{3}H]Ro 15-4513 binding to hippocampal membranes of α6-100RR (RR) and α6-100QQ (QQ) rats (mean ± standard error of the mean; RR, $n = 4$; QQ, $n = 5$), Difference in B_{max} between the rat lines: $P = .062$, two-tailed unpaired *t*-test.

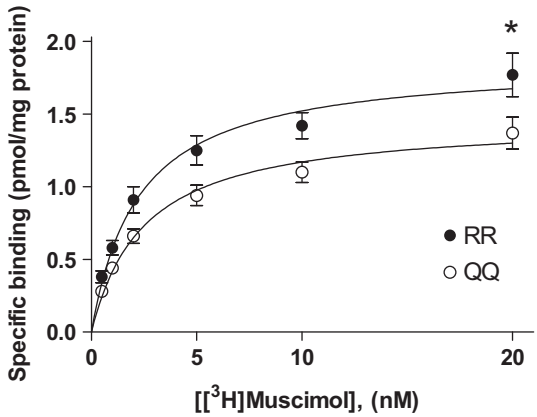

Fig. 6. Saturation analysis of [^{3}H]muscimol binding to cerebellar membranes of α6-100RR (RR) and α6-100QQ (QQ) rats (mean ± standard error of the mean; RR, $n = 7$; QQ, $n = 8$). *$P < .05$, significance of the difference in B_{max} between the rat lines, two-tailed unpaired *t*-test.

Discussion

Using two groups of Sprague–Dawley rats homozygous for $GABA_AR$ alleles α6-100R and α6-100Q, we found several differences between the groups in their cerebellar $GABA_ARs$ previously found between the AT and ANT rat lines: the affinity of cerebellar DZ-IS [^{3}H]Ro 15-4513 binding sites is higher to (1) [^{3}H]Ro 15-4513 and to (2) classic BZs in QQ than in RR animals; (3) the number of cerebellar high-affinity [^{3}H]muscimol binding sites is

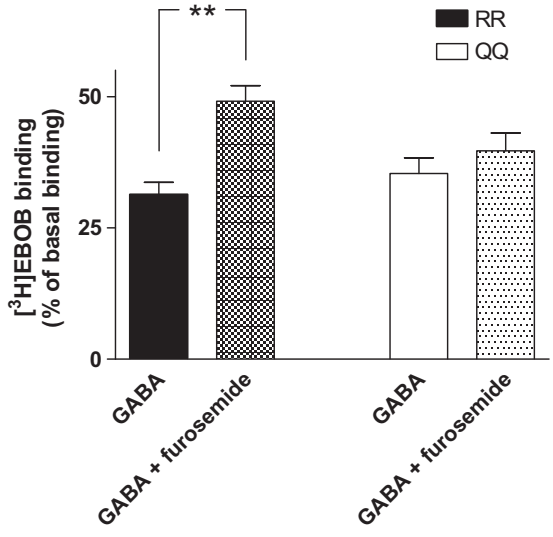

Fig. 7. Antagonizing effect of furosemide on GABA-induced displacement of [^{3}H]EBOB in cerebellar membranes from α6-100RR (RR) and α6-100QQ (QQ) rats (mean ± standard error of the mean; RR, $n = 11$; QQ, $n = 13$). **$P < .01$, significance of the difference between GABA-induced displacement and the antagonism of this displacement in RR rats, one-way ANOVA followed by Tukey's post hoc test.

lower in QQ than in RR animals; and (4) the GABA-antagonizing effect of furosemide on GABA-induced inhibition of [^{3}H]EBOB binding is lower in QQ than in RR animals. The affinity differences (1) and (2) have been previously published between alcohol-nonpreferring sNP rats homozygous for QQ and RR alleles (Sanna et al., 2003), between Sprague–Dawley rats with α6-100RR and α6-100QQ genotypes (Hanchar et al., 2005) and using α6-100R/Q-β2/3γ2 recombinant receptors (Hanchar et al., 2005; Korpi et al., 1993). The results clearly show that the differences (1, 2) are caused by the R to Q substitution in α6 residue 100.

As a surprise, this amino acid change affects association and dissociation kinetics of [^{3}H]Ro 15-4513 binding. Residue 100 in α6 (and the homologous His residue in α1) reside in loop A of the BZ binding pocket (Tan et al., 2007). Association and dissociation rates of [^{3}H]Ro 15-4513 binding appear to be very similar in α1-101H-β3γ2 and α6-100Q-β3γ2 receptors. It has been previously shown, that the α1-H101Q mutation in α1β2γ2 recombinant receptors increases [^{3}H]Ro 15-4513 binding affinity (Davies et al., 1998). In fact, α1-H101 substitutions F, K, R, E, Q, and C increase [^{3}H]Ro 15-4513 affinity while drastically decreasing flunitrazepam affinity (Davies et al., 1998, Wieland et al., 1992). These substitutions also affect efficacy of BZs, including Ro 15-4513, in α1β2γ2 receptors indicating the central role of the residue in BZ binding and function (Dunn et al., 1999). We performed [^{3}H]Ro 15-4513 association and dissociation measurements at 0°C. The association rates in vivo at 37°C would be much faster and although obviously slower in α6-100R receptors, equilibrium would be reached within minutes. Our data suggest that in vitro binding studies using [^{3}H]Ro 15-4513 as a ligand, 1-h incubation times at concentrations ≤5 nM at 0°C have obviously led to an underestimation of the number of cerebellar [^{3}H]Ro 15-4513 DZ-IS binding sites.

The other two AT/ANT line differences, (3) and (4), were present in RR/QQ rats, but the molecular basis of these differences is less clear. Cerebellar high-affinity [^{3}H]muscimol binding is mainly localized in cerebellar granule layer and associated with α6βγ2 and α6βδ receptor subtypes (Jones et al., 1997; Korpi et al., 2002; Mihalek et al., 1999). The higher B_{max} of high-affinity [^{3}H]muscimol binding therefore suggests a higher amount of α6βγ2 and/or α6βδ receptors in AT/RR than in ANT/QQ cerebellar granule cells. The basal level α6 mRNA expression has been shown to be higher in sNP rats with RR than with QQ genotype (Saba et al., 2005). This difference was suggested to be because of differences in α6 promoter sequences between RR and QQ genotypes (Saba et al., 2005). However, no difference has been found between AT and ANT rats in the number of cerebellar [^{3}H]Ro 15-4513 binding sites (Uusi-Oukari and Korpi, 1990) or in cerebellar granule cell tonic GABAergic currents (Valenzuela et al., 2005), and only a tendency (~10%) to higher level of α6 mRNA expression in AT than in ANT rats (Uusi-Oukari et al., 2000). However, the slow association of [^{3}H]Ro 15-4513 to DZ-IS binding sites in RR rats suggests that the B_{max} value of AT/RR rats might have been underestimated because the binding does not reach equilibrium within 1 h of incubation. Because of high BZ sensitivity, it is not possible to determine B_{max} of DZ-IS [^{3}H]Ro 15-4513 binding in QQ animals. It might be also difficult to see a 30% difference corresponding to difference in [^{3}H]muscimol binding in DZ-IS [^{3}H]Ro 15-4513 binding by determining total [^{3}H]Ro 15-4513 binding, because a 30% difference in 20–25% DZ-IS portion of total binding would make a 7% difference in B_{max} of total binding. In addition to a difference in α6 mRNA transcription, there may be differences in the efficiency between α6-100R and α6-100Q proteins to combine with other subunits to form α6βγ2 and/or α6βδ receptors.

The blunted furosemide action in antagonizing GABA-induced inhibition of [^{3}H]EBOB binding was also seen in QQ rats in the present study. The result suggests the involvement of residue 100Q on the mechanism(s) of blunted furosemide response in ANT/QQ rats. However, the inability to reproduce this line difference using α6-100Q-β3γ2 recombinant receptors (Mäkelä et al., 1996) suggests that in addition to α6-R100Q amino acid change there are other unidentified alteration(s) in ANT/QQ receptors necessary for the insensitivity to furosemide. There may also be $GABA_A$R-associated component(s) present in cerebellar granule cells needed for the ANT/QQ furosemide insensitivity not present in HEK cells used for recombinant receptor expression. Granule cell-specific posttranslational modification is also possible. Anyway, the data shown here suggest that the furosemide-insensitive property seems to be linked to α6-100Q allele.

There was a tendency for lower [^{3}H]Ro 15-4513 binding in hippocampal membranes of QQ rats when compared with RR rats. The result is in accordance with the hippocampal line difference between AT and ANT rats (Uusi-Oukari and Korpi, 1990). $GABA_A$R subunits are clustered in genome as clusters of 3–4 subunits (Darlison et al., 2005). The α6 gene is clustered with genes for α1, β2, and γ2 subunits, the latter three subunits forming the major $GABA_A$R subtype α1β2γ2 in the brain (Garrett et al., 1997; McKernan and Whiting, 1996). The α6-100Q genotype is strictly associated with various silent polymorphisms/single nucleotide polymorphisms (SNPs) in the other three $GABA_A$R subunit genes of the cluster (Congeddu et al., 2003). Therefore, it remains possible that SNPs in α1, β2, and γ2 subunit genes (that cosegregate with the α6-100Q genotype) might contribute to lower number of hippocampal [^{3}H]Ro 15-4513 binding sites in ANT/QQ than in AT/RR rats.

Although the results suggest that most of the differences in cerebellar $GABA_A$Rs are dependent on α6-R100Q polymorphism, it is possible that other polymorphisms found in the genes of the β2-α6-α1-γ2 cluster and linked to α6-R100Q polymorphism might play a role in some of the differences between RR and QQ rats. The R100Q

mutation is enriched in two alcohol-nonpreferring rat lines (sNP and Alko nonalcohol), suggesting that in addition to ethanol and BZ sensitivity to motor impairment the polymorphism and/or the associated SNPs of the cluster might be associated in alcohol preference and avoidance (Carr et al., 2003; Saba et al., 2001; Wong et al., 1996a). Genes in β2-α6-α1-γ2 cluster have also been associated to ethanol-related behaviors in humans. Several genetic association studies with human alcohol-dependent subjects suggest a role for genes in β2-α6-α1-γ2 cluster in the development of alcohol dependence (Chang et al., 2002; Loh and Ball, 2000; Loh et al., 1999; Radel et al., 2005; Sander et al., 1999).

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