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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