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Brain regional distribution of $GABA_A$ receptors exhibiting atypical GABA agonism: Roles of receptor subunits

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ABSTRACT

The major inhibitory neurotransmitter in the brain, γ-aminobutyric acid (GABA), has only partial efficacy at certain subtypes of $GABA_A$ receptors. To characterize these minor receptor populations in rat and mouse brains, we used autoradiographic imaging of *t*-butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS) binding to $GABA_A$ receptors in brain sections and compared the displacing capacities of 10 mM GABA and 1 mM 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP), a competitive GABA-site agonist. Brains from $GABA_A$ receptor α1, α4, δ, and α4 + δ subunit knockout (KO) mouse lines were used to understand the contribution of these particular receptor subunits to "GABA-insensitive" (GIS) [^{35}S]TBPS binding. THIP displaced more [^{35}S]TBPS binding than GABA in several brain regions, indicating that THIP also inhibited GIS-binding. In these regions, GABA prevented the effect of THIP on GIS-binding. GIS-binding was increased in the cerebellar granule cell layer of δ KO and α4 + δ KO mice, being only slightly diminished in that of α1 KO mice. In the thalamus and some other forebrain regions of wild-type mice, a significant amount of GIS-binding was detected. This GIS-binding was higher in α4 KO mice. However, it was fully abolished in α1 KO mice, indicating that the α1 subunit was obligatory for the GIS-binding in the forebrain.

Our results suggest that native $GABA_A$ receptors in brain sections showing reduced displacing capacity of [^{35}S]TBPS binding by GABA (partial agonism) minimally require the assembly of α1 and β subunits in the forebrain and of α6 and β subunits in the cerebellar granule cell layer. These receptors may function as extrasynaptic $GABA_A$ receptors.

1. Introduction

γ-Aminobutyric acid (GABA) A-type receptors are pentameric ligand-gated anion channels mediating inhibitory currents in the adult mammalian central nervous system. They are composed of 16 receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ and π) with different brain regional gene expression profiles (Wisden et al., 1992), forming the basis for $GABA_A$ receptor diversity. Several receptor subunit combinations (subtypes) can be distinguished by their affinities for various drugs (Olsen et al., 1990; Korpi and Lüddens, 1993; Sieghart, 1995; Mitchell et al., 2008). Receptor subtypes may also differ in their synaptic versus extrasynaptic location, conductance and opening time (Brickley et al., 1999; Birnir and Korpi, 2007; Mody, 2008).

t-Butylbicyclophosphorothionate (TBPS) is a picrotoxin-type $GABA_A$ receptor blocker, which is thought to bind inside the $GABA_A$ receptor ion channel. This view is suggested by experiments carried out using site-directed mutagenesis and cysteine accessibility methods (Xu et al., 1995; Bali and Akabas, 2007). Dissociation of [^{35}S]TBPS correlates well with the function of the ion channel measured by anion flux (Im and Blakeman, 1991). In general, [^{35}S]TBPS dissociates from its binding sites when agonists are applied and this effect can be inhibited by antagonists (Squires et al., 1983; Ticku and Ramanjaneyulu, 1984; Lüddens and Korpi, 1995). It is likely that [^{35}S]TBPS is dissociated from its channel binding sites due to agonist binding-mediated allosteric effects on the channel structure, but the effect of receptor desensitization for the dissociation, especially during long incubations, cannot be fully excluded. The [^{35}S]TBPS binding assay has been used as a simple biochemical assay of $GABA_A$ receptor function, even though it is an indirect method and the detailed binding mechanisms are still unknown.

Previous experiments with [^{35}S]TBPS have revealed a proportion of $GABA_A$ receptors displaying atypical allosteric coupling

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between the agonist and channel sites: GABA fails to induce full displacement of [^{35}S]TBPS, leaving the so-called GABA-insensitive [^{35}S]TBPS binding (GIS-binding) (Sinkkonen et al., 2001b). This binding component is detectable in brain sections by autoradiography. GIS-binding is enriched in the cerebellar granule cell layer and thalamic nuclei, two brain regions in which $GABA_A$ receptor subunits δ, α6 and α4 are enriched (Wisden et al., 1992; Pirker et al., 2000). Experiments with recombinant receptors have revealed that many $GABA_A$ receptor subtypes may contribute to GIS-binding (Sinkkonen et al., 2001a). Interestingly, $GABA_A$ receptors containing α6 subunit express more GIS-binding than α1 subunit-containing receptors, and mice lacking α6 have reduced GIS-binding (Sinkkonen et al., 2001a). Mice heterozygous for γ2 subunit deletion display increased GIS-binding, suggesting a role for dimeric αβ receptors (Sinkkonen et al., 2004a). In line with this observation, transgenic mice ectopically expressing extrasynaptic dimeric α6β $GABA_A$ receptors in the hippocampus have increased GIS-binding and increased tonic inhibition in that brain region (Wisden et al., 2002; Sinkkonen et al., 2004b). Additionally, these α6 transgenic mice have increased amount of $GABA_A$ receptors in the hippocampus, in which the competitive GABA-site agonist 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridine-3-ol (THIP, gaboxadol) is stronger than GABA (Saarelainen et al., 2008). GABA actually inhibits the THIP effect in [^{35}S]TBPS binding autoradiography of this mouse model and in electrophysiology of recombinant α6β3(δ) receptors. Thus, GABA is a partial agonist at the novel extrasynaptic receptors of this mouse line. δ KO mice do not have deficient forebrain GIS-binding (Sinkkonen et al., 2001a). The obvious hypothesis for the present study was that the α4 KO mice would have dramatically reduced levels of GIS-binding, similarly to the cerebellum of α6 KO mice.

In this study, we have characterized the capacities of high concentrations of GABA and THIP to displace [^{35}S]TBPS binding in a number of animal models using ligand autoradiography method. First, we studied the brain regional distribution of displacing capacities using rat brain sections. Then, we used mouse lines deficient in α1, α4, δ, or α4 and δ subunits, to see which of the subunits was responsible for the GIS-binding in the forebrain.

2. Methods

2.1. Animals

Male Wistar rats ($n = 9$, Harlan Netherlands B.V., Netherlands) were decapitated at 4 months of age and whole brains were carefully dissected out, rinsed in ice-cold saline and frozen on dry ice. Frozen brains were wrapped in plastic and stored at −80 °C until used.

Four different $GABA_A$ receptor subunit knockout mouse lines were used along with respective wild-type controls. The α1 subunit knockout mice (α1 KO; $n = 5$) and corresponding littermate wild-type controls (WT2; $n = 6$) were of a mixed C57BL/6J, FVB, and Strain 129S1/X1 genetic background and were created by interbreeding heterozygous α1 KO mice (Vicini et al., 2001). The α4 subunit knockouts (α4 KO; $n = 3$ for [^{35}S]TBPS autoradiography, $n = 6$ for [^{3}H]Ro 15-4513 autoradiography), δ subunit knockouts (δ KO; $n = 3$), combined α4 and δ subunit knockouts (α4 + δ KO; $n = 5$) and their wild-type controls (WT1; $n = 4$ for [^{35}S]TBPS autoradiography, $n = 6$ for [^{3}H]Ro 15-4513 autoradiography) were of a mixed C57BL6/J and Strain 129S1/X1 genetic background. They were created by first interbreeding α4 KO (Chandra et al., 2006) and δ KO (Mihalek et al., 1999) and then interbreeding the double heterozygous offspring. Mice were decapitated at the age of 2–3 months, and whole brains were carefully dissected out, rinsed in ice-cold saline and frozen on dry ice. The frozen brains were wrapped in plastic and stored at −80 °C until used.

All animals were group housed, given standard rodent chow and tap water *ad libitum*, and maintained on a 12-h alternating light/dark schedule, at the temperature of 20–22 °C. All procedures were approved by the Institutional Animal Use and Care committees of the University of Pittsburgh, the University of Helsinki, and the Southern Finland provincial government.

2.2. Ligand autoradiography

[^{35}S]TBPS autoradiography to examine brain regional distribution of GIS-binding was carried out as described in detail (Sinkkonen et al., 2001b). Frozen brains were cut to 14-μm frontal or horizontal sections using a Leica CM 3050 S cryostat. Sections were thaw-mounted to gelatin-coated object glasses and stored at −80 °C until autoradiography. Each object glass contained sections from all mouse groups to be compared, with the cutting order being balanced for the groups.

Sections were preincubated in an ice-water bath for 15 min in 50 mM Tris–HCl (pH 7.4) supplemented with 120 mM NaCl to remove traces of endogenous GABA, thus minimizing its effect on [^{35}S]TBPS binding kinetics. Basal [^{35}S]TBPS binding was performed in fresh incubation buffer of the above composition supplemented with 2 nM [^{35}S]TBPS (700–900 CPM/μl final incubation solution; PerkinElmer Life and Analytical Sciences, Boston, USA) at 20–22 °C for 90 min. Displacement of [^{35}S]TBPS binding was studied in the presence of various concentrations of GABA, THIP or GABA + THIP. After preliminary experiments with a range of saturating millimolar GABA (1–70 mM; Sigma Chemical Company, St. Louis, USA) and THIP (1–10 mM; THIP–HCl, H. Lundbeck A/S, Copenhagen, Denmark) concentrations, optimal concentrations for revealing interactions between GABA and THIP were found to be 10 mM GABA and 1 mM THIP, as reversal of THIP inhibition needed about 10-fold higher concentration of GABA. In the TBPS binding assay, THIP acts like a full agonist (Rabe et al., 2000). The nonspecific binding was determined in the presence of 100 μM picrotoxinin (Sigma). After incubation, to focus on the GIS-binding, the sections were subjected to a thorough washing step (three times 30 min in ice-cold, NaCl-free 10 mM Tris–HCl, pH 7.4). Sections were then dipped in ice-cold distilled water (0–4 °C) and dried under a fan at 20–22 °C. Then, the sections were exposed to Biomax MR films (Eastman Kodak, Rochester, USA) with plastic ^{14}C-microscale standards (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 2 days (basal binding conditions) to 3 weeks (other binding conditions).

The autoradiographic assay of [^{3}H]Ro 15-4513 binding was carried out as previously described (Mäkelä et al., 1997). Briefly, mouse brain sections were preincubated in an ice-water bath for 15 min in 50 mM Tris–HCl (pH 7.4) supplemented with 120 mM NaCl. Final incubation in the preincubation buffer was performed in the dark with 15 nM [^{3}H]Ro 15-4513 (PerkinElmer) at 0–4 °C for 60 min. The binding was also studied in the presence of 1 and 10 μM diazepam (Orion Pharma, Espoo, Finland; dissolved in dimethylsuphoxide) to reveal the α6 and α4 subunit-dependent diazepam-insensitive binding. Nonspecific binding was determined in the presence of 10 μM flumazenil (Hoffman-La Roche, Basel, Switzerland). It was at the background level (not shown). After incubation, the sections were washed in ice-cold incubation buffer (0–4 °C) twice for 60 s. Sections were then dipped into ice-cold distilled water, air-dried at room temperature, and exposed with plastic ^{3}H-microscale standards to Biomax MR films for 5 weeks.

Films of rat brain sections and [^{3}H]Ro 15-4513 experiment were quantified using MCID M5 image analysis devices and programs (Imaging Research Inc., St. Catharines, Ontario, Canada). Those of [^{35}S]TBPS experiments on mouse sections were first scanned using an EPSON Expression 1680 Pro scanner and EPSON Scan v. 1.11e program and then analysed with Scion Image analysis program (Scion Corporation, Frederick, Maryland, USA). The microscale standards were used as the reference and the resulting binding values were converted to radioactivity levels estimated for grey matter areas (nCi/g for ^{35}S and nCi/mg for ^{3}H). Images from representative films were produced by scanning the films using the same scanner and scanning program and Corel Paint Shop Pro Photo XI (version 11.20, Corel Corporation, Ottawa, Ontario, Canada) and Corel Draw X3.

2.3. Statistics

Specific [^{35}S]TBPS binding values were determined by subtracting the nonspecific binding values from the corresponding total binding values. Binding densities for each brain area were averaged from measurements from three to nine animals. Paired or standard *t*-test and one-way analysis of variance and Bonferroni post-hoc test were used when determining statistical significance of the differences between groups (Prism program, version 5.00, GraphPad Software, San Diego, CA).

3. Results

3.1. Brain regional distribution of [^{35}S]TBPS displacing capacities of 10 mM GABA and 1 mM THIP

Basal [^{35}S]TBPS binding was seen throughout the rat brain (Fig. 1). Picrotoxinin at 100 μM displaced almost all the [^{35}S]TBPS binding, leaving a negligible level of nonspecific binding. A saturating GABA concentration, 10 mM, inhibited the binding almost to the background levels in most brain areas. Significant binding was left in certain areas, such as the thalamus and granule cell layer of cerebellum, revealing the GIS-binding as shown previously (Sinkkonen et al., 2001b). When THIP was applied at 1 mM, a concentration which has been shown to inhibit [^{35}S]TBPS binding more effectively than 10 mM GABA in regions with GIS-binding and less effectively elsewhere (Saarelainen et al., 2008), a different binding pattern was revealed. THIP inhibited the binding to a lesser degree in most brain areas compared to GABA, although