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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.

Keywords

$GABA_A$ receptor; [^{35}S]TBPS autoradiography; THIP; Gaboxadol; Partial agonism; Thalamus; Cerebellum

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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 from 16 receptor subunits (α1-6, β1-3, γ 1-3, 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 Ramanjaneylu, 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 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 four 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 interations 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 two days (basal binding conditions) to three 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 its effect was greater in a few regions (Table 1). In the external layer of the cerebral cortex, the ventroposterolateral thalamus and granule cell layer of the cerebellum there was less [^{35}S]TBPS binding in the presence of THIP than GIS-binding (Table 1).

3.2. GABA-insensitive, THIP-sensitive [^{35}S]TBPS binding in different knockout mouse lines

Recently, Saarelainen et al. (2008) demonstrated that transgenic mice over-expressing the $GABA_A$ receptor α6 subunit gene ectopically in forebrain had increased GIS-binding in the hippocampus, and that THIP decreased [^{35}S]TBPS binding more efficiently than GABA. They also found that GABA competitively inhibited the effect of THIP on the binding in a concentration-dependent manner (i.e., high concentration of GABA reversed the THIP-induced inhibition of the GIS-binding). Recombinant dimeric α6β3 receptors expressed in HEK 293 cells possessed similar properties. In the present study, we wanted to explore which receptor subtype might be responsible for the GIS-binding detected in the native brain (Fig. 1). Since this binding is enriched in the thalamus and cerebellar granule cell layer, we concentrated on these brain regions. We used knockout mouse lines lacking the genes encoding for α1, α4, δ and α4 + δ subunits of the $GABA_A$ receptor (α1 KO, α4 KO, δ KO and α4 + δ KO, respectively) with respective wild-type controls, and performed [^{35}S]TBPS autoradiography with them.

High basal [^{35}S]TBPS binding was seen in all mouse lines (Fig. 2a, Fig. 3a). There were certain differences in the basal [^{35}S]TBPS binding between the wild-type and knockout mice (Table 2). In the thalamus, α4 and α4 + δ KO mice had significantly increased basal binding when compared to the wild-type mice, and in the δ KO mice the trend was similar ($p = 0.10$). In contrast, α1 KO mice had significantly decreased [^{35}S]TBPS binding in the thalamus as compared to the wild-type mice. In the granule cell layer of the cerebellum, δ KO and α4 + δ KO mice had increased basal binding, where as α4 KO and α1 KO mice had unaltered binding.

The wild-type mice displayed the same kind of GIS-binding profile as described earlier (Sinkkonen et al., 2001b). In wild-type mice, similar to rats (Fig. 1), 1 mM THIP inhibited significantly more [^{35}S]TBPS binding when compared to 10 mM GABA in the granule cell layer of the cerebellum and in the thalamus (Figs 2-3). Significantly less binding remained after application of THIP when compared to co-application of GABA and THIP (Figs 2b, 3b), which suggests that GABA acts as a partial agonist when compared to THIP. Also in the α4, δ and α4 + δ KO mice, THIP inhibited significantly more binding in the cerebellar granule cell layer than GABA (Fig. 2b), the reduced displacing capacity of GABA being the clearest in the δ subunit-deficient mice. The same was true for the thalamus of α4 KO mice, but not for that of the δ subunit-deficient mice, in which GABA and THIP exhibited similar inhibitions. Since the basal [^{35}S]TBPS was increased in these mice (Table 2), the amount of GIS-binding was increased in all of them, with the binding relative to the basal binding being unchanged or even slightly reduced as compared to wild-type mice.

To see how γ2 subunit-dependent binding sites were altered in the α4 KO brain, we performed labelling of the benzodiazepine sites with [^{3}H]Ro 15-4513 (Mäkelä et al., 1997). We found out that thalamic labelling was increased in α4 KO mice (30.8 ± 1.2 nCi/mg vs. 23.8 ± 0.5 nCi/mg, $p < 0.001$, *t*-test, mean ± SEM, n=6; Fig. 4). The α4 subunit-dependent diazepam-insensitive binding was almost absent (1.8 ± 0.1 nCi/mg vs. 0.7 ± 0.1 nCi/mg, $p < 0.001$, *t*-test, mean ± SEM, n=6).

Since the α1 KO mouse line derived from a different genetic background than the other KO mouse lines, wild-type littermate controls (WT 2) of the α1 KO mice were used. In α1 KO mice, the [^{35}S]TBPS binding profile in the thalamus differed from the other mouse lines. The basal binding was strongly reduced and the GIS-binding was at background level (Fig. 3). This indicates that α1 subunit is essential for the GIS-binding in almost all the forebrain areas. In

addition, GABA was significantly more effective than THIP (Fig. 3), and co-application of GABA and THIP inhibited the binding more than THIP alone (Fig. 3). In the cerebellar granule cell layer, the α1 KO mice did not differ from their wild-type controls, except for not revealing any statistically significant differences between the inhibitions by GABA, THIP and GABA + THIP.

There are two brain areas that merit a special note in the α1 KO brains, namely the reticular nucleus of thalamus and the CA4 region of hippocampus (Fig. 3a). The reticular nucleus of thalamus has a very low basal [^{35}S]TBPS binding (Table 1), but retains a high level of binding in the presence of 1 mM THIP (24 ± 4.5 % of basal, mean ± SEM, n=5) and becomes therefore clearly visible in the α1 KO brains. The low THIP sensitivity of this area might be due to the presence of α3- and non-α1 subunit-containing $GABA_A$ receptors (Ebert et al., 1994;Mozrzymas et al., 2007). The native α3 receptors obviously do not contribute to GIS-binding. The stratum oriens of the CA4 region retains some binding in the presence of 10 mM GABA (6.4 ± 1.5 % of basal, mean ± SEM, n=5), perhaps suggesting the presence of some unknown $GABA_A$ subtypes or e.g., “ectopic” α6 subunit expression. GIS-binding was present in the CA4 area of all genotypes.

4. Discussion

In this study we have demonstrated GABA-insensitive [^{35}S]TBPS binding to $GABA_A$ receptors in several brain regions of the rat and mouse: the cerebellar granule cell layer, various thalamic nuclei, cerebral cortex, caudate-putamen, colliculi and the CA4 area of the hippocampus. We also deciphered the effects of inactivating the genes encoding for α1, α4 and δ subunits of the $GABA_A$ receptor. The knockout mouse lines helped to understand the native $GABA_A$ receptor subtypes where GABA is weaker than THIP and may act like a partial agonist.

Previous electrophysiological findings on recombinant receptors (Storustovu and Ebert, 2006) and [^{35}S]TBPS autoradiography on brain regional distributions of GIS binding (Sinkkonen et al., 2001b) suggested that the reduced displacing capacity of GABA would be caused by $GABA_A$ receptors containing a δ subunit, two β subunits and two α subunits, either α4 (forebrain) or α6 (cerebellum) (see Introduction). The correlation between α4 subunit expression and GIS-binding in the forebrain (Sinkkonen et al., 2001b) and high THIP sensitivity and efficacy of the α4 subunit-containing $GABA_A$ receptors (Krogsgaard-Larsen et al., 2004) would have made them a likely population of receptors responsible for GIS-binding. Therefore, the present experiments with α4 KO and δ KO mouse brains were surprising, as the forebrain GIS-binding was not abolished: although its proportion of the basal binding was either reduced (α4 + δ KO), unaltered (δ KO) or even increased (α4 KO), it was clearly present in all these mouse lines, especially if we take into consideration the increase in the basal [^{35}S]TBPS binding in these mouse models (Table 2).

Preliminary data suggest that various compensations among the remaining $GABA_A$ receptor subunits take place in the thalamus of α4 KO mice [decreased δ subunit expression and transport to the cell membrane (Peng et al., 2007; Zhang et al., 2007), increased amounts of α1 and α2 subunits (Peng et al., 2007), with a subtle increase in γ2 subunit on the plasma membrane (Peng et al., 2007)], which suggest that other subunits than α4 are responsible for reduced displacing capacity of GABA and inhibition of THIP effects by GABA in the forebrain. We found here a 29% increase in thalamic γ2 subunit-dependent benzodiazepine-site labelling in α4 KO mice (Fig. 4), indicating normal availability of γ2 subunits. Based on these results, the native α4 subunit-containing receptors cannot have the similar GABA pharmacology as the structurally related α6 subunit-containing receptors in the cerebellum, although these receptor subtypes share many other selective pharmacological features, such as insensitivity to

benzodiazepine agonists and sensitivity to furosemide antagonism (Korpi et al., 1995; Wafford et al., 1996).

Against expectations, the GIS-binding the thalamic nuclei and elsewhere in the forebrain was abolished when the gene encoding for α1 subunit was inactivated (Fig. 3). In recombinant $GABA_A$ receptors, α1βγ2 receptors produce hardly any GIS-binding. In contrast, α1β receptors lacking γ2 subunit display significant GIS-binding (Sinkkonen et al., 2004a). Hence, a simple explanation for forebrain GIS-binding is the existence of dimeric α1β $GABA_A$ receptors in the native brain. This is supported by strongly increased and widespread forebrain GIS-binding in the γ2 subunit knockout heterozygous mice (Sinkkonen et al., 2004a). As shown earlier (Sinkkonen et al., 2001a), the δ KO mice displayed increased GIS-binding in the granule cell layer of the cerebellum, indicating that δ subunit is not compulsory for GIS-binding. Deleting the $GABA_A$ receptor α1 subunit did not affect the cerebellar GIS-binding, which strengthens the importance of the α6 subunit in GIS-binding in the granule neurons. Furthermore, the increased α1 subunit might also explain the increased thalamic GIS-binding in the α4 KO mice (see above).

Compensatory changes occurring in subunit knockout mouse models may complicate the interpretation of our binding data. In the thalamus of α1 KO mice, the $GABA_A$ receptor α4 subunit does not associate with gephyrin (Kralic, et al., 2006), a protein essential for synaptic clustering of $GABA_A$ receptors (Essrich, et al., 1998) even in the presence of the γ2 subunit and gephyrin. This suggests that the α4 subunit does not replace the missing synaptic α1 subunits. Immunoreactivity for the α3 and α4 subunits was increased (Kralic, et al., 2006). Our knowledge of receptor assembly is incomplete, which leaves us speculating on the precise subunit compositions and subcellular distributions of $GABA_A$ receptors with specific binding properties. Thus, the precise subunit composition of $GABA_A$ receptors formed in the thalamus of α1 KO mice is not known. Homomeric β3 $GABA_A$ receptors are known to produce [^{35}S] TBPS binding that is not displaced by GABA (Slany et al., 1995), and this is probably a consequence of lack of α/β subunit interfaces required for binding of GABA (see, Smith and Olsen, 1995). Homomeric β3 receptors do not bind GABA (Davies et al., 1997; Slany et al., 1995). [^{35}S]TBPS binding to β3 receptors is strongly inhibited by allosteric agonists, such as barbiturates, neurosteroids and propofol (Davies et al., 1997; Slany et al., 1995), similarly to GIS-binding to native receptors in the cerebellum and thalamus (Sinkkonen et al., 2001b). However, the role of β3 subunits in producing GIS-binding has been excluded by unaltered GIS-binding in the β3−/− mouse brains (Sinkkonen et al., 2001a). Finally, more generally, it is possible that the agonist and channel binding sites are not normally coupled in $GABA_A$ receptors where two β subunits are situated as neighbours, as suggested previously (Sinkkonen et al., 2004a). This hypothesis remains to be tested e.g. in recombinant $GABA_A$ receptors with known concatenated subunit configurations (see e.g., Kaur et al., 2009).

On the other hand, we think that the present results demonstrate brain regional differences in the efficacy of allosteric coupling between GABA and channel sites in specific receptor populations rather than in the amounts of receptors being unable to bind GABA, since GABA has to bind to the receptor in order to produce inhibition of the THIP effect that we observed. Although we cannot fully rule out the role of receptor desensitisation as a functional correlate to displacement of [^{35}S]TBPS binding by agonists, Saarelainen et al. (2008) found in recombinant receptors expressing α6β3±δ subunits that THIP-induced currents were greater than GABA-induced currents and that simultaneous application of THIP and GABA produced currents that approached in amplitude those induced by high GABA alone, which indicated differences in the agonist efficacy. Recombinant α1β and α6β receptors lacking γ2 subunit display significant GIS-binding (Sinkkonen et al., 2004a), indicating a deficient coupling between GABA and ion channel sites. The γ2+/− mice have a lack of γ2 subunits and an increased assembly of αβ-like receptors with a consequential increase in GIS-binding

(Sinkkonen et al., 2004a; Lorez et al., 2000). Now, an opposite situation can be speculated for the α1 KO mice as the neurons are lacking the most abundant α subunit and there would be a surplus of γ2 subunits. If the formation of dimeric αβ receptors is normally due to the shortage of γ2, the surplus of γ2 could switch the assembly kinetics of $GABA_A$ receptors towards negligible formation of dimeric αβ receptors and all other α subunits would be assembled in pentamers with γ2 subunits. Our data speak for the formation of GIS-binding in $GABA_A$ receptors assembled from only α1 or α6 and β subunits, being deficient of γ2 subunits.

One limitation in using the present ligand autoradiography procedure as the method for describing the functional state of $GABA_A$ receptors is that [^{35}S]TBPS used to label $GABA_A$ receptors may penetrate the cell membrane and reach also to the intracellular space. It is unclear whether the radioactivity seen in the autoradiographs represents functional $GABA_A$ receptors or internalized receptors or their early assembly products or degrading receptors with intact ion channels. The *in vitro* imaging of the maximal agonist efficacy at displacing [^{35}S]TBPS binding relies on millimolar concentrations of agonists, while the assumed extrasynaptic receptor pool normally faces much lower levels of "spillover" GABA (about 0.1-0.4 μM; Richerson and Wu, 2003; Tossman et al., 1986). The experiments with transgenic mice over-expressing α6 subunits in the hippocampal CA1 region indicate that the dimeric α6β receptors are functional *in situ* and in behaving animals (Sinkkonen et al., 2004b; Saarelainen et al., 2008). These transgenic mice show increased hippocampal GIS-binding and are exceptionally sensitive to THIP both *in vitro* and *in vivo*, indicating that new $GABA_A$ receptors are pharmacologically active. In line with our studies, the α1 KO mice have reduced sedative/hypnotic effects of THIP (Blednov et al., 2003). However, the α4 KO mice that have plenty of forebrain THIP-sensitive GIS-binding (the present study), are insensitive to ataxic, sedative and analgesic effects of THIP (Chandra et al., 2006). Also in the δ KO mice, hypnotic (Boehm et al., 2006) and electroencephalographic effects (Winsky-Sommerer et al., 2007) of THIP are reduced. Further studies are needed to assess the behavioural pharmacological significance of GIS-binding, which may well be different for the cerebellar and forebrain binding sites. For example, it would important to study the THIP sensitivity of GIS-binding in $GABA_A$ receptor β3 subunit-deficient mice, which are insensitive to hypnotic effects of THIP (Ugarte et al., 2000). The interest in the mechanisms of action of THIP is increasing as the drug may be clinically significant (see, Wafford and Ebert, 2006) and as it may be a useful tool in the pharmacological distinction between the synaptic γ2 subunit-containing $GABA_A$ receptors mediating fast phasic currents and the extrasynaptic often δ subunit-containing receptors mediating tonic "background" conductance (Farrant and Nusser, 2005).

In conclusion, we suggest that in the thalamus and other forebrain regions there are extrasynaptic α1β receptors (with and without δ subunits) that are responsible for THIP-sensitive GIS-binding. The α6 subunit-containing receptors mediate similar pharmacology with partial GABA agonism in the granule cell layer of the cerebellum. Based on these findings, there is a need for further study on minor $GABA_A$ receptor populations containing the most prevalent α1 subunit.

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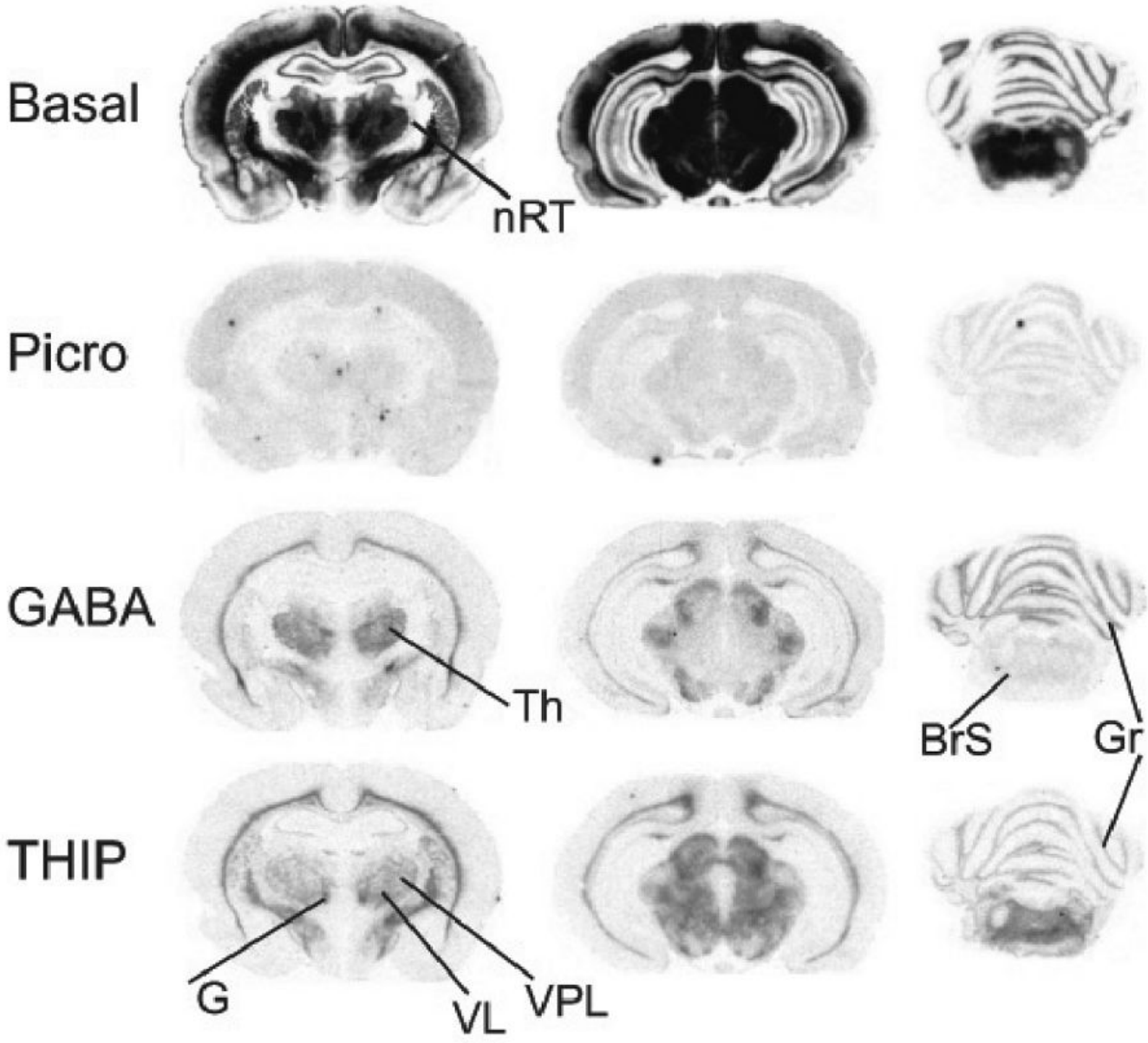

Fig. 1.

The effects of GABA and THIP on picrotoxinin-sensitive [^{35}S]TBPS binding in serial frontal sections of rat brain. *BrS*, brain stem; *G*, gelatinosus nucleus of thalamus; *Gr*, granule cell layer of cerebellum; *nRT*, reticular nucleus of thalamus; *Th*, thalamus; *VL*, ventrolateral thalamus; *VPL*, ventroposterolateral thalamus; SN, substantia nigra. Representative images were scanned and processed using identical scaling for brightness and contrast; the basal images were scanned from films after 2-day exposure and the other images from films after 21-day exposure.

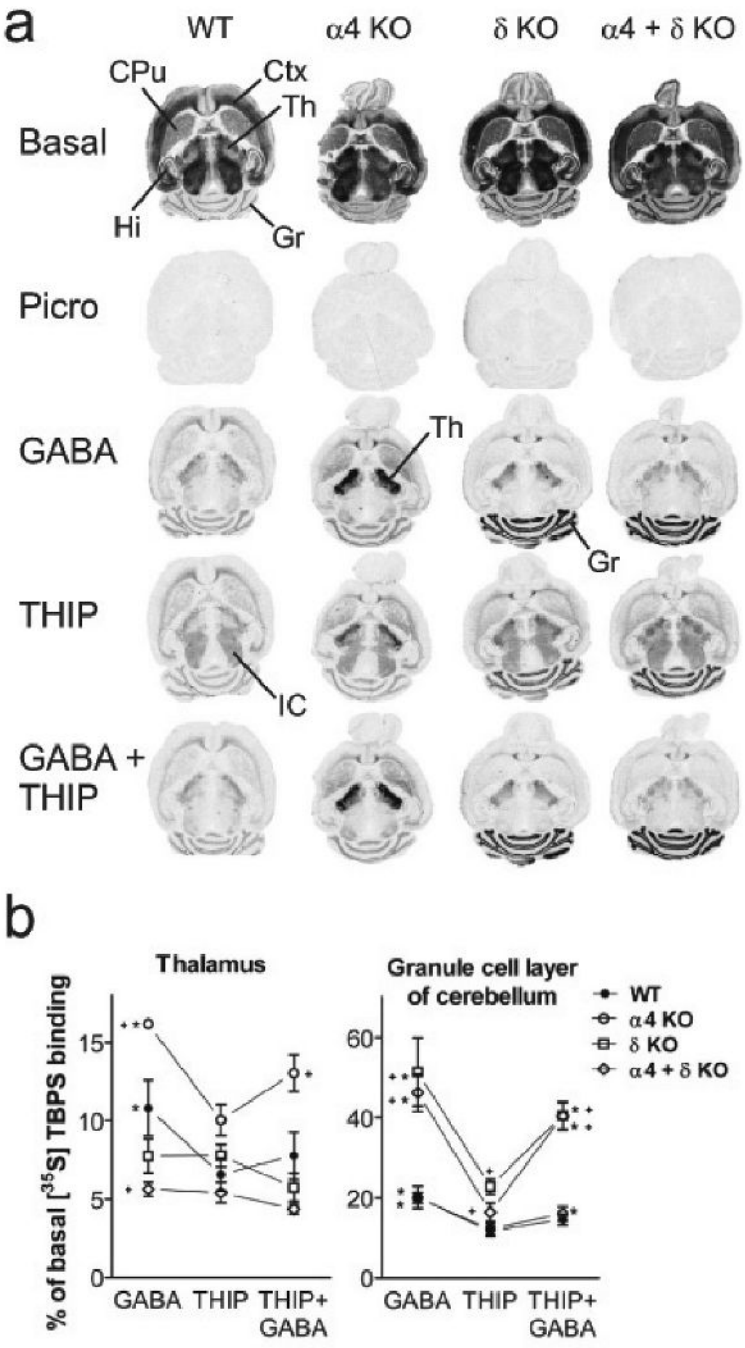

Fig. 2.

[^{35}S]TBPS binding and its modulation by GABA, THIP or both in horizontal sections of wild-type, α4 KO, δ KO and α4+δ KO mouse brains. **a**, Representative images were scanned and processed using identical scaling for brightness and contrast; the basal images were scanned from films after 2-day exposure and the other images from films after 21-day exposure. *Ctx*, cortex; *CPu*, caudate-putamen; *Gr*, granule cell layer of cerebellum; *Hi*, hippocampus; *IC*, inferior colliculus; *Th*, thalamus. **b**, Quantitative autoradiography results of the effects of 10 mM GABA, 1 mM THIP or both on [^{35}S]TBPS binding in the thalamus and granule cell layer of cerebellum of different mouse lines. Data are means ± SEM (n = 3 for α4 KO, δ KO, n = 4 for wild type and n = 5 for α4+δ KO mice), expressed as percent of the corresponding basal

[^{35}S]TBPS binding value. *$p < 0.05$ for the significance of the difference between GABA and THIP or between THIP and THIP + GABA (paired *t*-test). $^{+}$ $p < 0.05$ for the significance of the difference from the corresponding wild-type value (Bonferroni *t*-test).

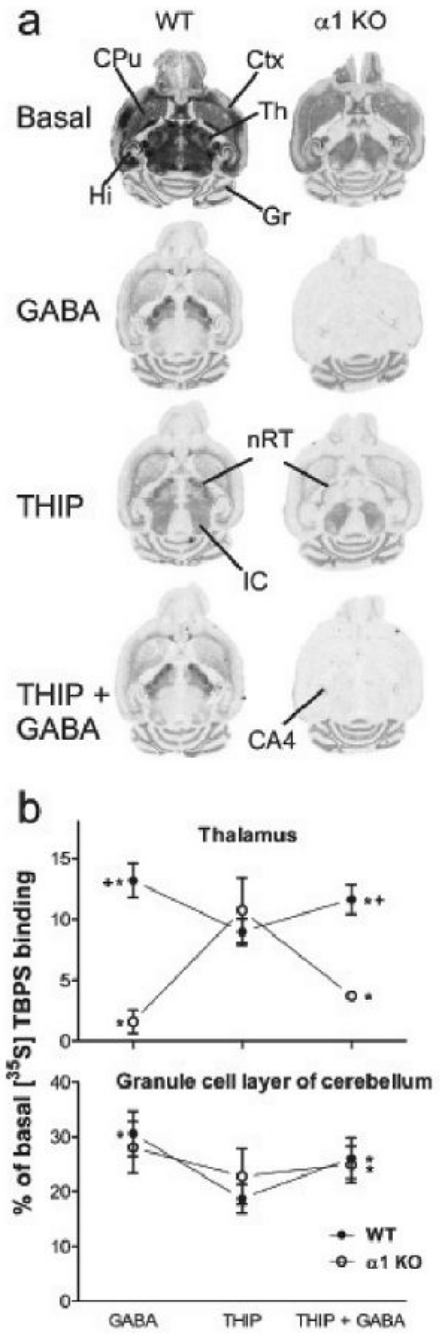

Fig. 3.

Modulation of [^{35}S]TBPS binding by GABA and THIP in α1 KO mice. **a**, Representative images of [^{35}S]TBPS binding and its modulation by 10 mM GABA, 1 mM THIP or both on wild-type and α1 KO mouse horizontal brain sections. *CA4*, CA4 area of the hippocampus; *Ctx*, cortex; *CPu*, caudate-putamen; *Gr*, granule cell layer of the cerebellum; *Hi*, hippocampus; *nRT*, reticular nucleus of the thalamus; *Th*, thalamus; *IC*, inferior colliculus. All the binding was abolished in the presence of 100 μM picrotoxinin as in Figs 1 and 2 (not shown). The representative images were scanned and processed using identical scaling for brightness and contrast, but the basal binding image was taken from films exposed only for 2 days rather than 21 days. **b**, Quantitative proportions of the [^{35}S]TBPS binding in the presence of 10 mM

GABA, 1 mM THIP or both in the thalamus and granule cell layer of cerebellum. Data are means ± SEM (n = 5 for α1 KO and n = 6 for wild-type mice) and expressed as percent of the corresponding basal [^{35}S]TBPS binding value. *$p < 0.05$ for the significance of the difference between GABA and THIP or between THIP and THIP + GABA (paired *t*-test). $^{+}$ $p < 0.05$ for the significance of the difference from the corresponding wild-type value (Bonferroni *t*-test).

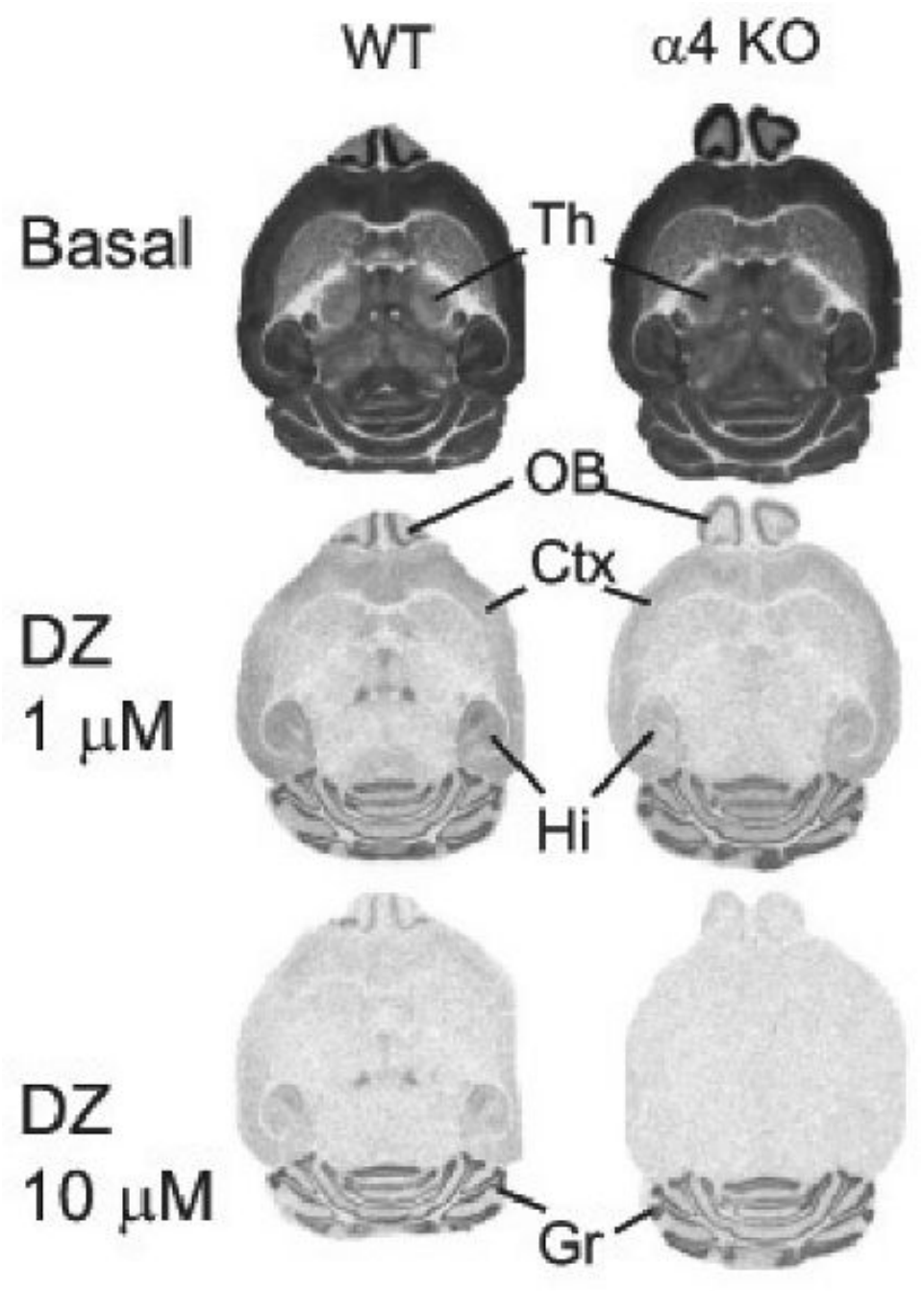

Fig. 4.

[^{3}H]Ro 15-4513 binding and its modulation by 1 and 10 μM diazepam (DZ) in α4 KO and wild-type brain sections. *Ctx*, cerebral cortex; *Gr*, granule cell layer of the cerebellum; *Hi*, hippocampus; *OB*, olfactory bulb; *Th*, thalamus.

Table 1

[^{35}S]TBPS binding and its modulation by GABA and THIP in frontal sections of the rat brain as revealed by quantitative autoradiography.

Brain area	Basal binding (nCi/mg)	GABA 10mM (% of basal)	THIP 1mM (% of basal)
Olfactory bulb	295±36	3.1±0.6	3.4±0.7
Cerebral cortex, external layer	543±66	1.3±0.2	1.1±0.2[b]
Cerebral cortex, internal layer	1026±73	0.9±0.1	1.2±0.2[a]
Anterior cortical amygdaloid nucleus	1840±261	1.1±0.2	2.0±0.2[c]
Medial amygdaloid nucleus	1535±198	1.0±0.2	2.3±0.4[b]
Anterior amygdaloid area	2049±219	1.1±0.2	2.0±0.3[b]
Caudate-putamen anterior	630±42	0.9±0.1	1.5±0.2[b]
Caudate-putamen posterior	523±23	1.1±0.1	1.7±0.1[b]
Globus pallidus	1704±158	0.7±0.1	2.6±0.2[c]
Globus pallidus-ventral pallidum	1641±164	0.8±0.1	2.5±0.2[c]
Globus pallidus-basal nucleus of Meynert	1395±94	0.8±0.1	2.8±0.2[c]
Nucleus vertical limb of diagonal band	1787±147	2.1±0.2	2.1±0.2
Nucleus horizontal limb of diagonal band	1819±150	1.6±0.1	2.5±0.3[a]
Lateral preoptic area	1527±167	1.2±0.1	1.6±0.3
Lateral hypothalamus	1375±166	0.7±0.1	1.6±0.3[b]
CA3 area of hippocampus	510±59	1.5±0.2	1.4±0.2
CA4 area of hippocampus	541±40	1.3±0.2	2.3±0.3[c]
Reticular nucleus of thalamus	82±9	4.3±2.6	20±6[b]
Gelatinosus nucleus of thalamus	1334±125	3.0±0.4	2.6±0.2
Ventrolateral thalamic nucleus	1049±105	3.4±0.6	2.6±0.3
Ventroposterolateral thalamic nucleus	907±88	4.2±0.5	3.0±0.5[b]
Medial geniculate nucleus	1030±29	2.6±0.2	2.8±0.2
Substantia nigra, reticular part	1487±115	0.8±0.2	1.8±0.1[b]
Paranigral nucleus	1028±123	1.0±0.2	3.0±0.7[b]
Oculomotor nucleus	1198±138	1.3±0.2	3.4±0.4[c]
Nucleus lateral lemniscus dorsal/ventral	1452±190	1.4±0.2	2.6±0.4[b]
Superficial grey layer of superior colliculus	1642±121	1.4±0.2	3.0±0.3[b]
Inferior colliculus	1701±119	1.2±0.1	2.4±0.2[c]
Olivary pretectal nucleus	1279±131	2.4±0.2	2.9±0.3[a]
Anterior pretectal area	1808±80	1.5±0.2	3.1±0.3[b]
Central gray	1076±93	1.0±0.1	2.1±0.3[a]
Brain stem	493±71	1.6±0.1	9.2±1.9[b]
Granule cell layer of cerebellum	281±26	7.1±0.6	4.6±0.4[b]

The values (mean ± SEM, n = 9) of basal binding are shown as nCi/mg, others are per cent values of the corresponding basal [^{35}S]TBPS binding.

[a] $p < 0.05$ for the significance of difference between the proportions of remaining [^{35}S]TBPS binding after application of THIP compared to GABA (paired *t*-test).

[b] $p < 0.01$ for the significance of difference between the proportions of remaining [^{35}S]TBPS binding after application of THIP compared to GABA (paired *t*-test).

[c] $p < 0.001$ for the significance of difference between the proportions of remaining [^{35}S]TBPS binding after application of THIP compared to GABA (paired *t*-test).

Table 2

Basal [^{35}S]TBPS binding in the thalamus and cerebellar granule cell layer in horizontal brain sections of $GABA_A$ receptor subunit knockout mice and their wild-type controls.

Mouse line	Thalamus	Granule cell layer
WT1	635±93	322±55
δ KO	923±113	654±38[b]
α4 KO	975±51[a]	364±37
α4+ δ KO	959±60[a]	677±46[b]
WT2	618±64	220±34
α1 KO	70±4[c]	226±31

Values are means ± SEM in nCi/g (n=4 for wild-type WT1, n=3 for δ KO and α4 KO, n=5 for α4 + δ KO and α1 KO, n=6 for wild-type WT2).

[a] $p < 0.05$ for the significance of the difference from the corresponding value of the wild-type control (*t*-test).

[b] $p < 0.01$ for the significance of the difference from the corresponding value of the wild-type control (*t*-test).

[c] $p < 0.001$ for the significance of the difference from the corresponding value of the wild-type control (*t*-test).