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ALTERED RECEPTOR SUBTYPES IN THE FOREBRAIN OF $GABA_A$ RECEPTOR δ SUBUNIT-DEFICIENT MICE: RECRUITMENT OF γ2 SUBUNITS

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Abstract—A $GABA_A$ receptor δ subunit-deficient mouse line was created by homologous recombination in embryonic stem cells to investigate the role of the subunit in the brain $GABA_A$ receptors. High-affinity [^{3}H]muscimol binding to GABA sites as studied by ligand autoradiography was reduced in various brain regions of $\delta^{-/-}$ animals. [^{3}H]Ro 15-4513 binding to benzodiazepine sites was increased in $\delta^{-/-}$ animals, partly due to an increment of diazepam-insensitive receptors, indicating an augmented forebrain assembly of γ2 subunits with α4 subunits. In the western blots of forebrain membranes of $\delta^{-/-}$ animals, the level of γ2 subunit was increased and that of α4 decreased, while the level of α1 subunits remained unchanged. In the $\delta^{-/-}$ forebrains, the remaining α4 subunits were associated more often with γ2 subunits, since there was an increase in the α4 subunit level immunoprecipitated by the γ2 subunit antibody. The pharmacological properties of *t*-butylbicyclophosphoro[^{35}S]thionate binding to the integral ion-channel sites were slightly altered in the forebrain and cerebellum, consistent with elevated levels of α4γ2 and α6γ2 subunit-containing receptors, respectively.

The altered pharmacology of forebrain $GABA_A$ receptors and the decrease of the α4 subunit level in δ subunit-deficient mice suggest that the δ subunit preferentially assembles with the α4 subunit. The δ subunit seems to interfere with the co-assembly of α4 and γ2 subunits and, therefore, in its absence, the γ2 subunit is recruited into a larger population of α4 subunit-containing functional receptors. These results support the idea of subunit competition during the assembly of native $GABA_A$ receptors.

Key words: knockout mouse line, quantitative autoradiography, western blotting, subunit level, immunoprecipitation, subunit assembly.

$GABA_A$ receptors are the primary mediators of fast inhibitory neurotransmission in the mammalian brain. The $GABA_A$ receptors are structurally heterogeneous, the putative pentameric receptors consisting of combinations of the 16 known subunits (α1–6, β1–3, γ1–3, δ, π, ε and θ) (Barnard et al., 1998; Hevers and Lüddens, 1998; Bonnert et al., 1999) at unknown stoichiometry. This heterogeneity provides the structural basis for differential targeting by receptor subtype-selective drugs to improve the treatment of, for example, insomnia, anxiety, epilepsy, and alcohol withdrawal.

Recent studies have revealed the importance of a few widely expressed subunits, the deficiency of which cannot be compensated for in the developing mouse brain. $GABA_A$ receptor γ2 and β3 subunit-deficient mouse lines are severely affected (Günther et al., 1995; Homanics et al., 1997), and thus, these subunits are critically needed. In contrast, the deficiency of α1, β2, α5, γ3, γ2L and α6 subunits does not produce any drastic phenotype (Culiat et al., 1994; Homanics et al., 1997; Jones et al., 1997; Homanics et al., 1999; Sur et al., 2001). There is no evidence for full compensation of a missing subunit by other subunits in any of these knockout models.

Even in the absence of an altered behavioral phenotype, the α6-deficient mice show altered cerebellar $GABA_A$ receptor pharmacology in a way that cannot be directly explained by the missing subunit (Mäkelä et al., 1997), but might be linked to structural compatibility of the subunits as the α6 subunit deficiency also causes a strong reduction in the amount of the δ subunit protein in spite of the normal subunit mRNA levels (Jones et al., 1997). The α6 subunits, but not other α variants, are thus critically needed for the δ subunits to assemble into native cerebellar granule cell $GABA_A$ receptors. In a δ subunit-deficient mouse line (Mihalek et al., 1999), the cerebellar $GABA_A$ receptor populations change (Tretter et al., 2001), with the most significant change

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Abbreviations: DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline; Ro 15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; TBPS, *t*-butylbicyclophosphorothionate.

being increased assembly of the γ2 subunits with α6 subunits, the level of which remains unchanged. This suggests full compensation of the missing δ subunit with the γ2 subunit in the cerebellar granule cell $GABA_A$ receptors of the $\delta^{-/-}$ mice.

Preliminary data also suggest that forebrain $GABA_A$ receptors may be qualitatively altered in the $\delta^{-/-}$ mice (Mihalek et al., 1999), but this cannot be due to increased α6βγ2 receptors since the α6 subunit is restricted to the cerebellar granule cells. In the forebrain, the δ subunit is often expressed in the same regions as the α4 subunit (Wisden et al., 1992) that is structurally and pharmacologically close to the α6 subunits. Therefore, if the δ subunit in the forebrain is selectively assembled with a certain α subunit, this subunit might well be the α4. In the present study, we tested the hypothesis of selective subunit assembly in forebrain $GABA_A$ receptors by studying the pharmacological profiles of recognition sites for GABA and benzodiazepine-site ligands and the changes in forebrain α4 and γ2 subunit levels and their co-assembly in the δ subunit-deficient mouse line (Mihalek et al., 1999). Furthermore, we studied the whole brain *in situ* pharmacologically using an ion-channel-site ligand (Mäkelä et al., 1997) to understand whether the receptor subtype alterations in the absence of δ subunit are significant enough to affect the allosteric modulation of the GABA and ion-channel-site coupling by other ligands.

EXPERIMENTAL PROCEDURES

Materials

t-Butylbicyclophosphoro[^{35}S]thionate ([^{35}S]TBPS) and tritium-labelled ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate ([^{3}H]Ro 15-4513) were purchased from NEN Division (Dreieich, Germany) and methylamine-[^{3}H]muscimol from Amersham (Buckinghamshire, UK) or NEN. Flumazenil (Ro 15-1788) was donated by F. Hoffmann-La Roche (Basle, Switzerland) and diazepam by Orion Pharmaceutica (Espoo, Finland). GABA and picrotoxinin were purchased from Sigma Chemical (St. Louis, MO, USA). Methyl-6,7-dimethoxy-4-ethyl-β-carboline (DMCM) was purchased from Research Biochemicals (Natick, MA, USA) and $ZnCl_2$ from Merck (Darmstadt, Germany). In the present study, the antibodies anti-peptide α1(1–9) (Zezula et al., 1991), anti-peptide γ2(319–366) (Tretter et al., 1997), and anti-peptide α4(379–421) (Bencsits et al., 1999) were used. The specificity of these affinity-purified antibodies has been demonstrated previously (Zezula et al., 1991; Tretter et al., 1997; Bencsits et al., 1999).

Production of animals

Targeted deletion of the δ subunit of the $GABA_A$ receptor in mouse embryonic stem cells and production of wild-type ($\delta^{+/+}$), heterozygous ($\delta^{+/-}$), and homozygous ($\delta^{-/-}$) mice are described in detail by Mihalek et al., (1999). The replacement-type DNA-targeting construct positioned a selectable marker gene in exon 4, upstream of the exons required for the putative transmembrane regions of this receptor subunit. This targeting event prevented the production of δ protein, thus producing a true null allele. The mice used for the present studies were the F_2 generation on a mixed C57BL/6J×strain 129Sv/SvJ genetic background.

Animals with targeted alleles of both α6 and δ subunits were created by breeding δ chimeric males with F_2 $\alpha6^{-/-}$ females (Homanics et al., 1997). Double-heterozygous animals produced from this mating were used to establish breeding pairs yielding animals of the nine different possible genotypes. Breeding pairs were then established with these animals to yield four different true-breeding lines: $\alpha6^{+/+}$, $\delta^{+/+}$; $\alpha6^{+/+}$, $\delta^{-/-}$; $\alpha6^{-/-}$, $\delta^{+/+}$; and $\alpha6^{-/-}$, $\delta^{-/-}$. Breeding pairs were selected to minimize inbreeding.

Animals were genotyped by Southern blot analysis for the δ and α6 loci as described (Homanics et al., 1997; Mihalek et al., 1999). All mouse lines used in the present study were normal in their gross behavior and did not have shortened life span. The experimental protocols were approved by the institutional animal use and care committees of the University of Turku, the University of Pittsburgh and the University of Vienna.

Preparation of brain cryostat sections

Adult (about 3 months old) male mice were killed by decapitation and whole brains were rapidly dissected out and frozen on dry ice. For autoradiography, 14-μm horizontal or frontal serial sections were cut from 13 $\delta^{+/+}$, 13 $\delta^{-/-}$ and three $\delta^{+/-}$ brains using a Microm cryostat, thaw-mounted onto gelatin-coated object glasses, and stored frozen under desiccant at −20°C. Another set of four brains/line was sectioned from the double-knockout series of mouse lines. All experiments were carried out in parallel fashion with respect to mouse lines, eliminating any day-to-day variation in receptor assays between the lines.

Ligand autoradiography

The autoradiographic procedures for determining brain regional density of [^{3}H]Ro 15-4513, [^{3}H]muscimol, and [^{35}S]TBPS binding were as described by Mäkelä et al. (1997). In brief, sections were preincubated in an ice–water bath for 15 min in 50 mM Tris–HCl (pH 7.4) supplemented with 120 mM NaCl in the [^{3}H]Ro 15-4513 autoradiographic assay, and in 0.31 M Tris–citrate (pH 7.1) in the [^{3}H]muscimol assay. In [^{35}S]TBPS assays, endogenous GABA, which interferes with the determination of α6 subunit pharmacology (Korpi and Lüddens, 1993), was removed by preincubating the sections in an ice–water bath for 3×10 min in 50 mM Tris–HCl supplemented with 1 mM ethylenediamine tetraacetic acid (pH 7.4).

The final incubation in the respective preincubation buffer was performed with 6 nM [^{35}S]TBPS at room temperature for 90 min, assays with 20 nM [^{3}H]muscimol at 0–4°C for 30 min, and assays with 10 nM [^{3}H]Ro 15-4513 at 0–4°C for 60 min. Effects of diazepam, DMCM and Zn^{2+} ions in the presence of 0.5 μM GABA were tested on [^{35}S]TBPS binding. Displacement of [^{3}H]Ro 15-4513 binding was studied in the presence of 100 μM diazepam. After incubation, sections were washed 3×15 s or 2×30 s in an ice-cold incubation buffer in [^{35}S]TBPS and [^{3}H]Ro 15-4513 or in [^{3}H]muscimol assays, respectively. Sections were then dipped into distilled water, air-dried under a fan at room temperature, and exposed with plastic [^{3}H]- or [^{14}C]methacrylate standards to Kodak Biomax MR films for 1–8 weeks, producing non-saturated images on the films. Non-specific binding was determined with 10 μM flumazenil (Ro 15-1788), 20 μM picrotoxinin and 100 μM GABA in [^{3}H]Ro 15-4513, [^{35}S]TBPS and [^{3}H]muscimol assays, respectively. Images from representative autoradiography films were scanned, processed with Adobe Photoshop 4.0 and CorelDraw 9.0 programs, and printed for figures.

The concentrations of [^{3}H]muscimol (20 nM) and [^{3}H]Ro 15-4513 (10 nM) were equal to the dissociation constants for a range of recombinant and native $GABA_A$ receptors (Pritchett et al., 1989; Lüddens et al., 1990; Pritchett and Seeburg, 1990; Wisden et al., 1991; Dämgen and Lüddens, 1999). Therefore, the autoradiographic images should represent the density rather than affinity of binding sites. All the autoradiographic assays have been shown to be sensitive to alterations in receptor subunit levels in the $\alpha6^{-/-}$ mouse model (Mäkelä et al., 1997).

Analysis of autoradiographs

Autoradiography films were quantitated using AIS image analysis system (Imaging Research, St. Catharines, ON, Canada) as described (Mäkelä et al., 1997). Binding densities for each brain area were averaged from measurements of one to three sections/brain. The standards exposed simultaneously with brain sections were used as reference with the resulting binding values given as radioactivity levels estimated for gray matter areas (nCi/mg for ^3H and nCi/g for ^{14}C).

Quantitative immunoblot analysis

Membranes from a total of six $\delta^{+/+}$ and $\delta^{-/-}$ forebrains were isolated individually and equal amounts (7 μg) of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in different slots of the same 10% polyacrylamide gel (Jechlinger et al., 1998). Proteins were blotted to polyvinylidene difluoride membranes and detected by subunit-specific antibodies α1(1–9) (Zezula et al., 1991), α4(379–421) (Bencsits et al., 1999) and γ2(319–366) (Tretter et al., 1997). Secondary antibodies [$F_{(ab')2}$ fragments of goat anti-rabbit IgG, coupled to alkaline phosphatase; $F_{(ab')2}$ fragments of goat anti-mouse IgG, coupled to alkaline phosphatase (Axell, Westbury, NY, USA)] were visualized by the reaction of alkaline phosphatase with CSPD™ (Tropix, Bedford, MA, USA) and the chemiluminescent signal was quantified by densitometry of Kodak X-omat S films with the DocuGel 2000i gel documentation system using RFLP scan software (MWG-Biotech, Ebersburg, Germany). The linear range of the detection system was established by determining the antibody response to a range of antigen concentrations following immunoblotting. The experimental conditions were designed such that immunoreactivities obtained in the assay were within this linear range, thus permitting a direct comparison of the amount of antigen applied per gel lane between samples.

To test for equal protein loading, in some experiments, a monoclonal anti-β-actin antibody (monoclonal anti-actin; Amersham) was included in the antibody solution and the amounts of endogenous β-actin were quantitatively determined in the same slots analogous to $GABA_A$ receptor subunits. Protein loading was comparable in different slots and referring the data to the amounts of β-actin did neither change the results nor reduce variability.

To immunoprecipitate the $GABA_A$ receptor subunits, the receptors were first solubilized from forebrain membranes of $\delta^{+/+}$ and $\delta^{-/-}$ mice using a deoxycholate buffer (0.5% deoxycholate, 0.05% phosphatidylcholine, 10 mM Tris–HCl, pH 8.5, 150 mM NaCl, 0.3% phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 0.7 μg/ml pepstatin). Then, the receptors were precipitated by subunit-specific antibodies (Tretter et al., 1997) and equal amounts of protein per slot were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins transferred to polyvinylidene difluoride membranes were labelled with digoxygenated primary antibodies and detected by anti-digoxigenin-alkaline phosphatase F_{ab} fragments (Roche Molecular Biochemicals) and the chemiluminescence substrate DSPD™ (Tropix) as described above.

Statistical analyses

The significance of the differences between the $\delta^{+/+}$, $\delta^{-/-}$ and $\delta^{+/-}$ mouse groups and between two population means were assessed with the GraphPad Prism program by using one-way analysis of variance (ANOVA) followed by Newman–Keuls post-hoc test or by using Student's *t*-test, respectively.

RESULTS

[^{3}H]Muscimol-labelled GABA sites in the brain of mutant $\delta^{-/-}$ mice

The GABA binding agonist [^{3}H]muscimol, known to have especially high affinity to δ subunit-containing receptors (Quirk et al., 1995), was used as a radioligand to determine the brain regional changes of GABA binding sites in δ subunit-deficient mice. The binding was drastically diminished in the forebrain regions of the $\delta^{-/-}$ mice when compared to the $\delta^{+/+}$ mice, most conspicuously in the thalamus (Fig. 1; Table 1). Also the hippocampus, caudate-putamen and cerebral cortex had significantly less binding in the $\delta^{-/-}$ mice. Some binding

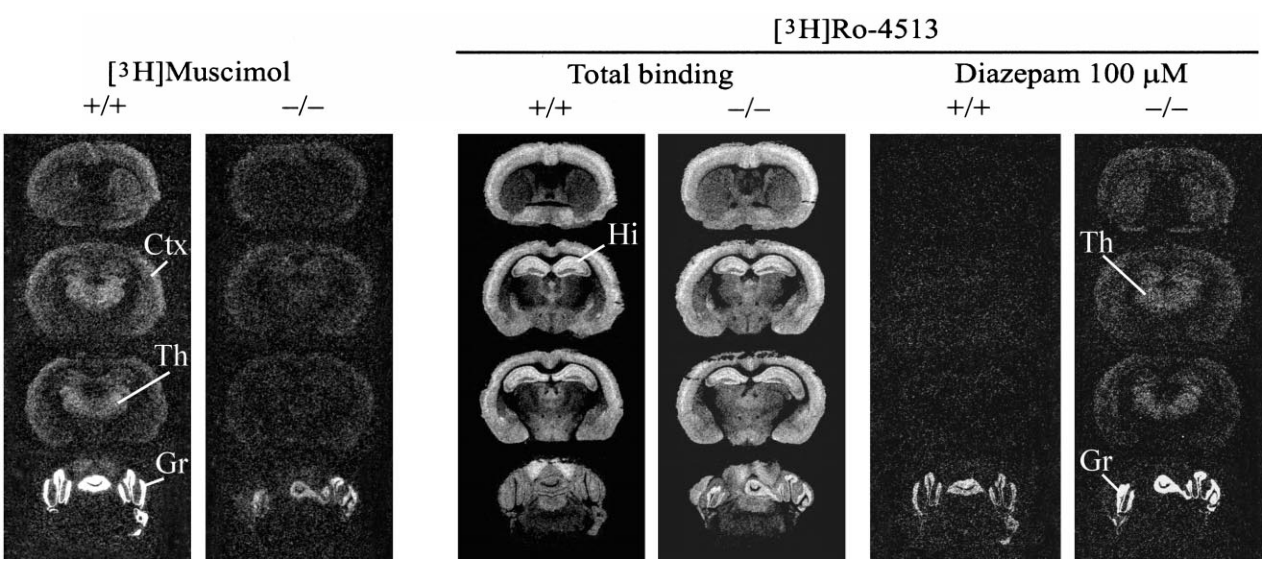

Fig. 1. Autoradiographic distribution of $GABA_A$ receptor binding sites in the frontal sections of $\delta^{-/-}$ (–/–) and wild-type $\delta^{+/+}$ (+/+) mouse brains. Total GABA sites were labelled by 20 nM [^{3}H]muscimol, the non-specific binding in the presence of 100 μM GABA being at the film background level. Total flumazenil-sensitive benzodiazepine sites were labelled by 10 nM [^{3}H]Ro 15-4513; diazepam-insensitive binding is also depicted. Ctx, cerebral cortex; Gr, cerebellar granule cell layer; Th, thalamus; Hi, hippocampus.

Table 1. Quantitative autoradiography of GABA-agonist sites labelled with [^{3}H]muscimol in various brain regions of wild-type $\delta^{+/+}$, heterozygous $\delta^{+/-}$ and homozygous mutant $\delta^{-/-}$ mice

Brain region	Mouse genotype		
	$\delta^{-/-}$	$\delta^{+/-}$	$\delta^{+/+}$
Thalamus	3.0 ± 0.7***	8.0 ± 1.1*	10.0 ± 1.5
Hippocampus	3.8 ± 0.5**	4.2 ± 0.2*	5.2 ± 0.7
Caudate-putamen	2.7 ± 0.4***	5.1 ± 0.5*	6.3 ± 0.9
Cerebral cortex	4.7 ± 0.7***	7.2 ± 0.9	7.6 ± 1.3
Cerebellum, granule cell layer	23 ± 3***	37 ± 1.4**	51 ± 7
Cerebellum, molecular layer	5.4 ± 0.4	5.3 ± 0.8	6.4 ± 1.1

Brain horizontal sections were incubated with 20 nM [^{3}H]muscimol in the presence and absence of 100 μM GABA. Autoradiographic films were processed and GABA-sensitive binding was quantified against radioactivity standards. Data (nCi/mg) are mean ± S.D. of four to eight animals in each group. ANOVA yielded significant [$F(2,17) = 11$–58, $P = 0.01$] differences between the genotypes for all brain regions, except for the cerebellar molecular layer [$F(2,17) = 3.2$, $P = 0.07$]. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, for the statistical significances of the difference from the wild-type $\delta^{+/+}$ values (Tukey–Kramer test).

remained in the cerebellar granule cell layer, indicating that there are other than δ-containing receptors that have high affinity to [^{3}H]muscimol.

Pharmacological specificity of the benzodiazepine-site binding in the brain regions of mutant $\delta^{-/-}$ mice

[^{3}H]Ro 15-4513 binding was widespread, as expected, due to the high affinity of [^{3}H]Ro 15-4513 to all $GABA_A$ receptors with benzodiazepine sites (Hevers and Lüddens, 1998) in the brains of both $\delta^{-/-}$ and $\delta^{+/+}$ mice (Fig. 1; Table 2), and completely displaceable by the benzodiazepine-site antagonist flumazenil (data not shown). The total binding level was significantly increased in the thalamus, caudate-putamen, cerebral cortex and cerebellum, but not in the hippocampus.

Diazepam-insensitive [^{3}H]Ro 15-4513 binding is considered to be the hallmark of cerebellar α6 (Lüddens et al., 1990) or forebrain α4 (Wisden et al., 1991; Benke et al., 1997) subunit-containing $GABA_A$ receptors. This component, determined in the presence of 100 μM diazepam, was small in all brain regions other than the cerebellar granule cell layer in the wild-type animals (Table 2; Fig. 1). However, it was increased in the $\delta^{-/-}$ mouse brains (Table 2; Fig. 1). Although the high concentration of diazepam may have underestimated the proportion of diazepam-insensitive [^{3}H]Ro 15-4513 binding, this increase was smaller than the increase in total [^{3}H]Ro 15-4513 binding.

Small, but statistically significant, differences between the $\delta^{-/-}$ and $\delta^{+/+}$ mice were also found in the total and diazepam-insensitive [^{3}H]Ro 15-4513 binding in the cerebellar molecular layer, although this brain region has undetectable α6 and δ gene expression (Wisden et al., 1992). This result may have been caused by ligand binding to ectopic granule cells in the molecular layer and/or by leakage of the ligand from the adjacent strongly labelled granule cell layer.

$GABA_A$ receptor subunit levels and co-precipitation in the forebrains of $\delta^{-/-}$ mice

Analyses of forebrain membranes from both $\delta^{-/-}$ and wild-type mice revealed that the γ2 subunit is signifi-

Table 2. Quantitative autoradiography of benzodiazepine sites labelled with [^{3}H]Ro 15-4513 in various brain regions of wild-type $\delta^{+/+}$ and homozygous mutant $\delta^{-/-}$ mice

Brain region	Mouse genotype	
	$\delta^{-/-}$	$\delta^{+/+}$
Total binding		
Thalamus	19 ± 1.4*	14 ± 2.1
Hippocampus	34 ± 0.8	34 ± 1.2
Caudate-putamen	16 ± 1.2**	11 ± 0.6
Cerebral cortex	39 ± 0.2*	33 ± 2.7
Cerebellum, granule cell layer	58 ± 0.6***	31 ± 0.5
Cerebellum, molecular layer	20 ± 1.4**	15 ± 0.7
Binding in the presence of 100 μM diazepam		
Thalamus	3.7 ± 0.2***	1.1 ± 0.2
Hippocampus	2.0 ± 0.2***	1.0 ± 0.2
Caudate-putamen	3.5 ± 0.3***	0.9 ± 0.1
Cerebral cortex	3.1 ± 0.5***	0.9 ± 0.1
Cerebellum, granule cell layer	30 ± 2.8***	13 ± 0.4
Cerebellum, molecular layer	1.6 ± 0.1***	0.8 ± 0.1

Serial brain frontal sections were incubated with 10 nM [^{3}H]Ro 15-4513 in the presence and absence of 10 μM flumazenil or 100 μM diazepam. Autoradiographic films were processed and quantified against radioactivity standards. Data (nCi/mg) are mean ± S.D. of three to four animals in both groups. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, for the statistical significances of the difference from the wild-type $\delta^{+/+}$ values (Student's *t*-test).

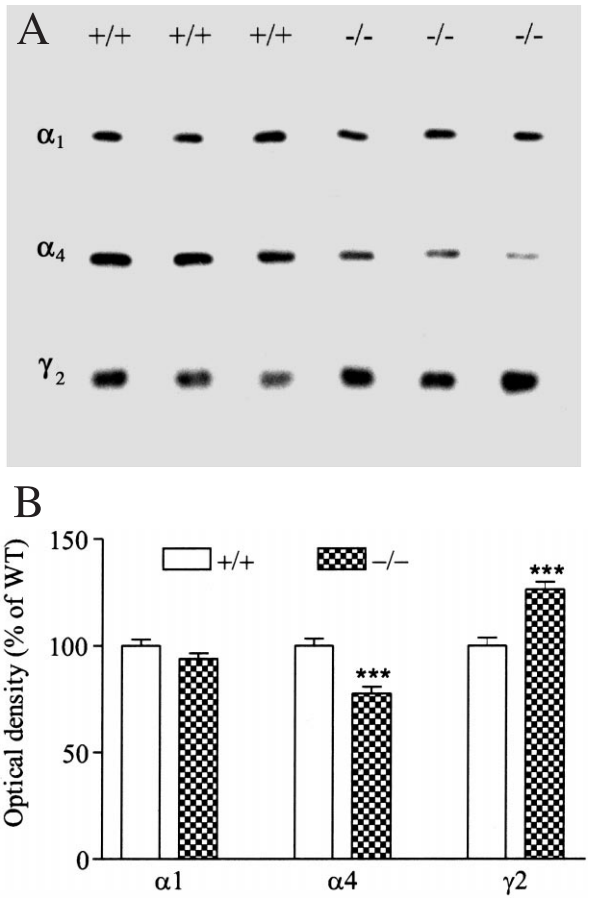

Fig. 2. Quantification of $GABA_A$ receptor α1, α4 and γ2 subunit proteins in the forebrain membranes of δ subunit-deficient (−/−) and wild-type (+/+) mice by western blot analysis. (A) Representative blots of the α1, α4 and γ2 subunit proteins. (B) Quantitative results of the western blots. The values are optical densities ± S.E.M. determined from fluorographs by image analysis for six mice, a value for each animal being the mean of the triplicate determinations, from both mouse lines. They are expressed as percentages of the mean subunit level found in $\delta^{+/+}$ membranes. ***$P < 0.001$, for the statistical significance of the difference from the wild-type (wt) values (Student's *t*-test).

cantly increased in the knockout animals (Fig. 2). Although the diazepam-insensitive component of the total benzodiazepine binding was also increased (Table 2), the α4 subunit that is responsible for this binding component in the forebrain was significantly reduced in the knockout animals (Fig. 2). The concentration of the α1 subunit remained unaltered in the forebrain of the $\delta^{-/-}$ mice.

To verify that the absence of the δ subunit increases the population of forebrain $GABA_A$ receptors with both γ2 and α4 subunits, we immunoprecipitated solubilized γ2 subunit-containing receptors with a γ2 antibody. The antibody pulled down higher amounts of γ2 (143 ± 8% of the wild-type values, which were 100 ± 6%, mean ± S.E.M., $n = 3$, $P < 0.05$) and α4 subunits (145 ± 8% vs. 100 ± 3%, $P < 0.01$) in the $\delta^{-/-}$ than $\delta^{+/+}$ mouse forebrain extracts. There was no significant difference between the mouse lines in the level of the α1 subunit co-precipitated with γ2 (119 ± 12% vs. 100 ± 4%, $P > 0.2$) in the $\delta^{-/-}$ and $\delta^{+/+}$ forebrains, respectively.

Ion-channel-site binding in the brain of $\delta^{-/-}$ mice

To assess whether the receptor subtype-dependent alterations in the GABA- and benzodiazepine-site bindings described above cause alterations in the function of $GABA_A$ receptors in the $\delta^{-/-}$ mice, we analyzed basal [^{35}S]TBPS binding to $GABA_A$ receptor ionophores and characterized and quantified several allosteric drug actions (Olsen et al., 1990; Mäkelä et al., 1997) using the [^{35}S]TBPS binding assay in serial sections of homozygous and wild-type brains. Basal [^{35}S]TBPS binding was significantly increased in the thalamus, caudate-putamen and cerebellar granule cell layer of the mutants as compared to wild-type brains (Table 3, Fig. 3). However, in comparison with the alterations in the GABA- and benzodiazepine-site bindings, the ion-channel-site binding is relatively little changed in various brain regions of the $\delta^{-/-}$ mice.

To reveal possible changes in the allosteric modulation of ion-channel sites, we first studied their coupling to GABA sites. In the presence of low exogenous GABA (0.5 μM), [^{35}S]TBPS binding was similar between the genotypes except for the cerebellar granule cell layer, in which $\delta^{-/-}$ mutants displayed a higher binding ($P < 0.001$)(Table 3; Fig. 3). Considering the higher basal binding in the mutants, this result suggests slightly increased GABA sensitivity of the mutants in the thalamus and caudate-putamen.

Allosteric modulation of the GABA and ion-channel coupling was studied in the presence of 0.5 μM GABA. The benzodiazepine-site agonist diazepam (1 and 30 μM) facilitated the GABA-induced decrease of [^{35}S]TBPS binding more in the thalamus and less in the cerebellar molecular layer of the mutants (Table 3; Fig. 3). The benzodiazepine-inverse agonist DMCM (1 μM) reversed the GABA-inhibition of [^{35}S]TBPS binding in all brain regions. It was more efficient in the caudate-putamen and less efficient in the cerebellar granule cell layer of the $\delta^{-/-}$ than $\delta^{+/+}$ mice. A high concentration of DMCM, which is considered to act agonistically [30 μM, (Stevenson et al., 1995)], was more efficient in the thalamus and cerebellar granule cell layer of the mutants. Zinc ions (10 and 100 μM), known to potently affect δ subunit-containing $GABA_A$ receptors (Saxena and Macdonald, 1996), enhanced the GABA-induced inhibition of [^{35}S]TBPS binding similarly in the forebrain of $\delta^{-/-}$ and $\delta^{+/+}$ mice. Only in the cerebellum, its effect was reduced in the mutant mice.

Pharmacological alterations in $\delta^{+/-}$ animals

[^{3}H]Muscimol binding revealed a significant decrease in GABA recognition sites in all analyzed brain regions of heterozygous $\delta^{+/-}$ mice compared to wild-type mice, except for the cerebellar molecular layer and cerebral cortex (Table 1). [^{3}H]Ro 15-4513 binding sites of the heterozygous animals were only slightly increased in comparison with those of the wild-type animals: a significant increase ($P < 0.01$) was found in the cerebellar granule cell (47 ± 6, $n = 4$, vs. 31 ± 6 nCi/g, $n = 8$,

Table 3. Quantitative autoradiography of picrotoxinin-sensitive $GABA_A$ receptor ionophore sites labelled with [^{35}S]TBPS in various brain regions of wild-type $\delta^{+/+}$ and homozygous mutant $\delta^{-/-}$ mice

Brain region	Mouse genotype	
	$\delta^{-/-}$	$\delta^{+/+}$
Basal binding (nCi/g)		
Thalamus	321 ± 56*	245 ± 17
Hippocampus	151 ± 8	157 ± 26
Caudate-putamen	153 ± 11***	116 ± 8
Cerebral cortex	287 ± 49	296 ± 59
Cerebellum, granule cell layer	283 ± 33*	236 ± 20
Cerebellum, molecular layer	97 ± 5	103 ± 20
GABA, 0.5 μM (nCi/g)		
Thalamus	236 ± 28	214 ± 12
Hippocampus	100 ± 10	98 ± 15
Caudate-putamen	105 ± 14	98 ± 13
Cerebral cortex	138 ± 31	141 ± 18
Cerebellum, granule cell layer	175 ± 3***	115 ± 7
Cerebellum, molecular layer	49 ± 14	47 ± 4
GABA+diazepam, 1 μM (% of GABA)		
Thalamus	54 ± 6*	65 ± 4
Hippocampus	47 ± 6	47 ± 6
Caudate-putamen	60 ± 10	59 ± 10
Cerebral cortex	54 ± 13	46 ± 9
Cerebellum, granule cell layer	57 ± 7	65 ± 11
Cerebellum, molecular layer	67 ± 9	60 ± 3
GABA+diazepam, 30 μM (% of GABA)		
Thalamus	44 ± 3*	51 ± 2
Hippocampus	40 ± 5	41 ± 4
Caudate-putamen	47 ± 6	51 ± 7
Cerebral cortex	52 ± 14	44 ± 16
Cerebellum, granule cell layer	44 ± 6	53 ± 10
Cerebellum, molecular layer	60 ± 2**	46 ± 7
GABA+DMCM, 1 μM (% of GABA)		
Thalamus	90 ± 8	88 ± 5
Hippocampus	148 ± 4	137 ± 14
Caudate-putamen	129 ± 8*	110 ± 10
Cerebral cortex	184 ± 18	170 ± 21
Cerebellum, granule cell layer	103 ± 2**	127 ± 10
Cerebellum, molecular layer	177 ± 31	159 ± 42
GABA+DMCM, 30 μM (% of GABA)		
Thalamus	67 ± 5*	75 ± 5
Hippocampus	105 ± 8	103 ± 6
Caudate-putamen	103 ± 4	94 ± 14
Cerebral cortex	150 ± 21	129 ± 19
Cerebellum, granule cell layer	51 ± 3***	81 ± 8
Cerebellum, molecular layer	108 ± 6	115 ± 13
GABA+Zn^{2+}, 10 μM (% of GABA)		
Thalamus	79 ± 3	82 ± 4
Hippocampus	72 ± 6	73 ± 4
Caudate-putamen	89 ± 7	82 ± 8
Cerebral cortex	102 ± 25	90 ± 12
Cerebellum, granule cell layer	91 ± 8**	71 ± 5
Cerebellum, molecular layer	90 ± 5*	74 ± 4
GABA+Zn^{2+}, 100 μM (% of GABA)		
Thalamus	62 ± 11	55 ± 7
Hippocampus	39 ± 4	35 ± 2
Caudate-putamen	47 ± 3	43 ± 7
Cerebral cortex	49 ± 11	43 ± 4
Cerebellum, granule cell layer	72 ± 5***	44 ± 6
Cerebellum, molecular layer	68 ± 11**	36 ± 8

Brain sections were incubated with 6 nM [^{35}S]TBPS in the presence and absence of 10 μM picrotoxinin. Autoradiographic films were processed and quantified against radioactivity standards. Data are mean ± S.D. of four $\delta^{-/-}$ and five $\delta^{+/+}$ animals. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, for the statistical significances of the difference from the wild-type $\delta^{+/+}$ values (Student's *t*-test).

mean ± S.D.) and molecular layers (17 ± 6 vs. 10 ± 1) and the caudate-putamen (10.8 ± 0.7 vs. 8.4 ± 1.1). No statistically significant ($P > 0.05$) differences were found between heterozygous and wild-type animals in the thalamus, hippocampus and cerebral cortex (data not shown). [^{35}S]TBPS binding was not studied in heterozygous animals, but it should be noted that the homozygous mutants did not differ from the wild-type animals in the cerebellar molecular layer, hippocampus or cerebral cortex (Table 3).

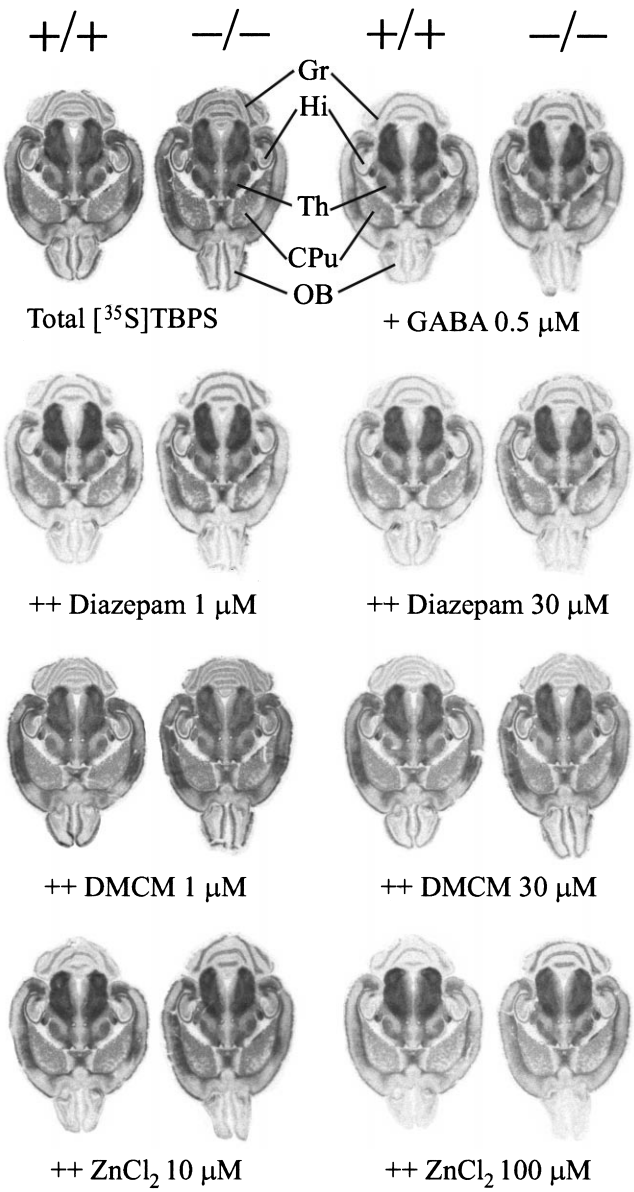

Fig. 3. Picrotoxinin-sensitive [^{35}S]TBPS binding to representative horizontal brain sections from $GABA_A$ receptor δ subunit-deficient (−/−) and wild-type (+/+) mice. The results show the effect of a low concentration (0.5 μM) of GABA and the effects of various other ligands at the concentrations specified in the presence of GABA. Gr, cerebellar granule cell layer; Hi, hippocampus; Th, thalamus; CPu, caudate-putamen; OB, olfactory bulb.

Effects of the deficiencies of δ and α6 subunits on benzodiazepine and GABA sites

To compare the alterations observed in the present study on δ knockout brains with those found in the α6 knockout brains (Mäkelä et al., 1997) that are also functional δ knockouts in the cerebellar granule cells, we also studied the global double-knockouts of the δ and α6 subunits. The autoradiographic images show that total [^{3}H]Ro 15-4513 binding was selectively reduced in the cerebellar granule cell layer of $\alpha6^{-/-}$ animals, while increased in $\delta^{-/-}$ animals (Fig. 4). A similar situation was observed in this brain region in the presence of diazepam: the forebrain [^{3}H]Ro 15-4513 binding profile was strongly altered in the presence of diazepam, the diazepam-insensitive binding being selectively increased in the absence of δ subunits. [^{3}H]Muscimol binding was generally reduced in the forebrain of the $\delta^{-/-}$ mice. The α6 subunit apparently contributes even more to the [^{3}H]muscimol binding, as the cerebellar granule cell layer binding was totally absent in $\alpha6^{-/-}$ brains which lack only 80% of the native δ protein. The double knockouts, $\alpha6^{-/-}\delta^{-/-}$, exhibited enhanced [^{3}H]Ro 15-4513 binding and reduced [^{3}H]muscimol binding in the forebrain, and very low levels of total [^{3}H]Ro 15-4513 and [^{3}H]muscimol binding in the cerebellum. These animals had no cerebellar and increased forebrain diazepam-insensitive [^{3}H]Ro 15-4513 binding.

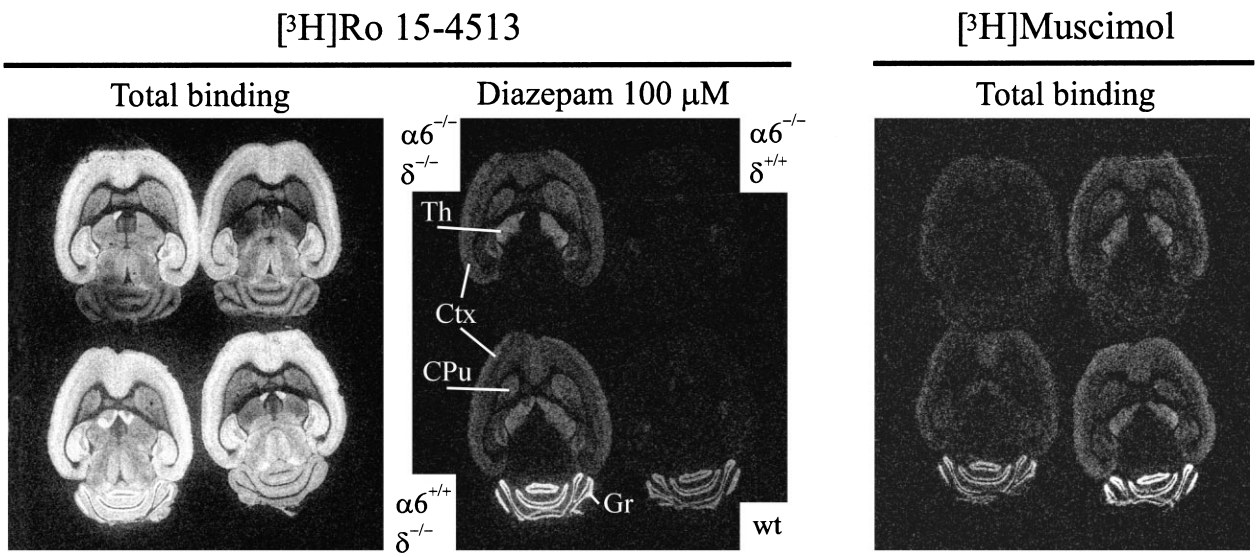

Fig. 4. Flumazenil-sensitive [^{3}H]Ro 15-4513 binding and GABA-sensitive [^{3}H]muscimol binding in brain sections from the wild-type (wt) $\alpha6^{+/+}\delta^{+/+}$, single-knockout $\alpha6^{+/+}\delta^{-/-}$ and $\alpha6^{-/-}\delta^{+/+}$, and double-knockout $\alpha6^{-/-}\delta^{-/-}$ mice. Data from one slide/condition, each representing serial horizontal sections of all genotypes (locations marked on the middle slide), are shown to illustrate total and diazepam-insensitive benzodiazepine sites and GABA sites. Three additional sets of four brains yielded identical results for each genotypes. Th, thalamus; Ctx, cerebral cortex; CPu, caudate-putamen; Gr, cerebellar granule cell layer.

DISCUSSION

The analyses of autoradiographic images for GABA-agonist sites and for benzodiazepine sites revealed both quantitative and qualitative differences between the $\delta^{-/-}$ and wild-type $\delta^{+/+}$ mice, indicative of selective alterations in the $GABA_A$ receptor subunit combinations in the absence of the δ subunit. This change in forebrain receptor subunit composition was directly demonstrated by immunoprecipitation experiments coupled with western blotting of the γ2 and α4 subunits, whose co-assembly was enhanced in the absence of the δ subunit. The study also proves that the δ subunits assemble into native receptors in all brain regions having significant δ subunit mRNA expression (Wisden et al., 1992), analogous to its assembly in heterologously expressed δ-containing receptors (Hevers et al., 2000). In addition, results obtained with heterozygous $\delta^{+/-}$ animals suggested a minor gene–dosage effect of the δ subunit gene on the binding profiles.

The binding sites for [^{3}H]Ro 15-4513 and [^{3}H]muscimol studied here in native preparations showed dissimilar alterations in the absence of the δ subunits. The binding of the GABA-agonist-site ligand [^{3}H]-muscimol was strongly reduced, while that of the benzodiazepine-site ligand [^{3}H]Ro 15-4513 was increased in several brain regions. The reduction in the [^{3}H]-muscimol-labelled GABA sites is in agreement with previous findings using immunoprecipitation (Quirk et al., 1995), which demonstrated that δ subunit-containing $GABA_A$ receptors have an exceptionally high equilibrium binding affinity to muscimol, and with the high functional sensitivity to GABA in heterologously expressed receptors containing the δ subunit (Saxena and Macdonald, 1996). However, [^{3}H]muscimol binding was not completely eliminated in δ subunit-deficient brains. The cerebellar α6 subunit, known to have a high agonist sensitivity as well (Korpi and Lüddens, 1993), is responsible for most of the cerebellar high-affinity [^{3}H]muscimol binding, as the α6 subunit-deficient mice are completely devoid of this binding component in autoradiography (Mäkelä et al., 1997)(Fig. 4). These data suggest that all cerebellar [^{3}H]muscimol binding is linked to the α6 subunit, either directly by being part of the sensitive receptor, or indirectly by preserving the δ subunit expression. Since muscimol is not subtype-selective in functional tests or in binding assays using heterologous expression systems, the present data suggest that [^{3}H]muscimol autoradiography under the employed conditions does not assess the brain regional distribution of all native $GABA_A$ receptors, but that it works best for δ and α6 subunit-containing receptors.

Since a γ subunit is obligatory for the formation of a benzodiazepine site (Pritchett et al., 1989) and the δ subunit fails to substitute for a γ subunit in this respect (Quirk et al., 1995; Jechlinger et al., 1998; Bencsits et al., 1999), the increase in [^{3}H]Ro 15-4513 binding in $\delta^{-/-}$ mice suggests that the number of $GABA_A$ receptors containing a γ subunit increases in the $\delta^{-/-}$ mice. The elevation of the γ2 subunit protein in the forebrain of $\delta^{-/-}$ mice, as predicted by ligand autoradiography, was confirmed by western blotting. An elevated proportion of the [^{3}H]Ro 15-4513 binding was insensitive to diazepam in $\delta^{-/-}$ mice, suggesting that the α subunit companion of δ is largely α4 in the forebrain and α6 in the cerebellum, and that in the absence of δ these two subunits assemble with γ2 subunits to create additional benzodiazepine binding sites in the $\delta^{-/-}$ mice. Increased association of γ2 and α4 subunits in the forebrain samples was confirmed by immunoprecipitation, as has the associa-

tion of γ2 and α6 subunits in the cerebellum (Tretter et al., 2001). However, since the level of α4 subunit protein was actually decreased, the rescue of α4 subunit-containing receptors by γ2 in the forebrain can have been only partial in the $\delta^{-/-}$ mice. In the cerebellum, the rescue is complete, because the level of α6 subunit is not reduced in the $\delta^{-/-}$ mice (Tretter et al., 2001). As the diazepam-sensitive [^{3}H]Ro 15-4513 binding in the forebrain, i.e., non-α4 receptors, increased in the $\delta^{-/-}$ mice as well, other α subunits with which δ co-localizes in the brain, such as α1, are likely to be normally present in part of the δ subunit-containing receptors (Bencsits et al., 1999; Sur et al., 1999). This proportion seems to be small and/or completely rescued by the γ2 subunit, since there was no alteration in the level of the α1 subunit in the knockouts and there was no statistically significant increase in the amount of α1 subunit pulled down by the γ2 subunit in the $\delta^{-/-}$ forebrains. In the cerebellum, the diazepam-sensitive [^{3}H]Ro 15-4513 binding was also increased, but this can be accounted for by increased α1 subunit level in the knockouts (Tretter et al., 2001). Our data suggest a preferential assembly of the δ subunits with α6 and α4 subunits. This has been experimentally proven in α6 subunit-deficient mice (Jones et al., 1997), but remains to be studied in α4 subunit-deficient forebrains as such mice become available.

The most likely mechanism for the receptor subtype alterations described here centers on the interaction of various subunits during receptor subunit assembly, i.e., in the absence of the δ subunit, more γ2 subunits assemble into functional receptors. This provokes the question of how assembly is regulated in the brain. Little is known of this process for $GABA_A$ receptors, except that α subunits and especially their N-terminal domains are obligatory (Connolly et al., 1996; Tretter et al., 1997; Taylor et al., 2000). Two scenarios exist to explain our results, namely, it is possible that the concentration of δ subunit exceeds that of the γ2 subunit, or that the δ subunit has a higher probability than the γ2 subunit in assembling with α4 and α6 subunits. In both scenarios, δ and γ2 subunits compete with each other during assembly into functional receptors in neurons, a process which can efficiently limit the number of receptor subtypes (subunit combinations) produced. However, we failed to demonstrate any preference of the γ2 subunit and/or exclusion of the δ subunit in the formation of recombinant α1β3γ2/δ $GABA_A$ receptors, or any preference of the δ subunit and/or exclusion of the γ2 subunit in the formation of recombinant α4β3γ2/δ and α6β3γ2/δ receptors in human embryonic kidney 293 cells (Hevers et al., 2000). In simultaneous transfection, both subunits are assembled into functional receptors based on their selective electrophysiological and pharmacological properties, though on a reduced expression level in comparison with transfection of γ2 alone with α and β3 subunits. Further studies on subunit competition in primary neuronal populations are warranted, since neurons, but not fibroblasts, have molecular mechanisms, such as clustering proteins (Chen et al., 2000; Kneussel et al., 2000), that might be needed for the selective assembly of subunits.

Since there are many different $GABA_A$ receptor subtypes in the brain, and since the loss of even the most common subtype containing α1 and β2 subunits can be compensated (Sur et al., 2001), it was of interest to study further consequences of the receptor subtype changes on the brain regional pharmacology in the δ-deficient mice. Picrotoxinin-sensitive ion-channel sites labelled with [^{35}S]TBPS can be used as an autoradiographic assay to detect changes in brain regional pharmacology after targeted disruption of receptor subunits (Mäkelä et al., 1997). These sites were less affected in the $\delta^{-/-}$ mice than the GABA or benzodiazepine sites. Basal [^{35}S]TBPS binding was slightly increased in the cerebellar granule cell layer, thalamus and caudate-putamen. Using [^{35}S]TBPS binding as a biochemical assay for $GABA_A$ receptor function (Squires et al., 1983; Im and Blakeman, 1991), we tested whether pharmacological features that are associated with δ subunit-containing receptors are altered in $\delta^{-/-}$ mice. Zn^{2+} affects most strongly heterologously expressed δ-containing $GABA_A$ receptors (Saxena and Macdonald, 1994), in line with the diminished effect of Zn^{2+} on [^{35}S]TBPS binding in the $\delta^{-/-}$ mouse cerebella. However, as the effect of zinc was similar in all other brain regions, the apparent proportion of δ-containing receptors is small, poorly labelled with [^{35}S]TBPS, or other subunit combinations are as sensitive to Zn^{2+} as the δ subunit-containing ones. The forebrain α4-containing receptors are insensitive to benzodiazepine agonists (Wisden et al., 1991; Benke et al., 1997). Diazepam potentiated the GABA-inhibition of [^{35}S]TBPS binding in the thalamus of $\delta^{-/-}$ mice more than that in wild-type mice, pointing to a supplemental increase in other than α4-containing benzodiazepine sites, in keeping with increased diazepam-sensitive [^{3}H]Ro 15-4513 binding in the thalamus. The β-carboline benzodiazepine-site ligand DMCM acts as an inverse agonist at low micromolar concentrations (1 μM) on non-α4/α6 receptors and as an agonist through a different binding site (Wingrove et al., 1994) at higher micromolar concentrations (30 μM) (Stevenson et al., 1995). This potentiation is independent of the type of α variant, but it is more easily detectable in benzodiazepine-insensitive receptors (α4 and α6). The inverse agonistic action in most brain regions was similar in both mouse lines (Fig. 3) with the exception of decreased efficacy in the cerebellar granule cell layer and a marginally increased efficacy in the caudate-putamen, the former hinting towards higher α6:α1 ratios in the $\delta^{-/-}$ mice. In the same mouse line, the agonistic action of DMCM was enhanced, i.e., it more efficaciously reduced the [^{35}S]TBPS binding in the cerebellar granule cell layer and thalamus. The residual binding remaining in the presence of GABA plus 30 μM DMCM [about 90 and 160 nCi/g in the cerebellar granule cell layer and thalamus, respectively (cf. Table 3)] was similar in quantity in both mouse lines, while the DMCM-inhibited components (mostly α6 and α4) were much greater in the mutant mice. Thus, the data on the ion-channel binding site indicate that the $\delta^{-/-}$ mice have an increased pool of receptor populations pharmacologically defined as α6 and α4 subunit-containing. However, since the α4 subunit level is actually decreased in the forebrain of the

$\delta^{-/-}$ mice (Fig. 2), it is possible that the δ subunit interferes with the detection of high-affinity binding of [^{35}S]TBPS to α4 subunit-containing receptors in the wild-type animals. In summary, in the $\delta^{-/-}$ mice, there are only small alterations in the allosteric actions of ligands whose effects have been shown to be dependent on the δ subunit in recombinant receptors.

The subunit interplay that we describe in the present study may have physiological consequences, as δ or γ2 subunit-containing $GABA_A$ receptors differ in their pharmacological properties, subcellular targetings and maximal GABA-induced currents (Saxena and Macdonald, 1994; Ducic et al., 1995; Zhu et al., 1996; Nusser et al., 1998). However, it should be noted that heterozygous $\delta^{+/-}$ mice had only small changes in their brain regional pharmacology as compared to wild-type animals, which indicates that receptor subtype profiles may be rather constant in spite of significant changes in the levels of δ subunit expression. Interestingly, the $\delta^{-/-}$ animals have behaviorally shown a selective insensitivity to neurosteroids (Mihalek et al., 1999). The mechanism of this insensitivity remains unknown, but it may at least partly be attributed to the emergence and differential subcellular localization of the novel γ2 subunit-containing receptors.

CONCLUSION

The alterations in ligand recognition sites in the brains of mice deficient in the $GABA_A$ receptor δ subunit suggest that the remaining $GABA_A$ receptor subunits readily assemble into functional receptors with differing pharmacology. The δ subunit preferentially assembles with α6 in the cerebellum and with α4 in the forebrain. In the $\delta^{-/-}$ mice, the γ2 subunit protein, whose assembly is normally reduced by δ, is recruited into $GABA_A$ receptors, as illustrated by the increased diazepam-insensitive benzodiazepine binding and elevated γ2 subunit levels. The data thus supports the idea of selective subunit assembly based on subunit availability and their competition in assembly mechanisms.

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