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# Cerebellar γ-Aminobutyric Acid Type A Receptors: Pharmacological Subtypes Revealed by Mutant Mouse Lines

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## SUMMARY

The vast molecular heterogeneity of brain γ-aminobutyric acid type A ($GABA_A$) receptors forms the basis for receptor subtyping. Using autoradiographic techniques, we established the characteristics of cerebellar granule cell $GABA_A$ receptors by comparing wild-type mice with those with a targeted disruption of the α6 subunit gene. Cerebellar granule cells of $\alpha6^{-/-}$ animals have severe deficits in high affinity [$^3$H]muscimol and [$^3$H]SR 95531 binding to GABA sites, in agonist-insensitive [$^3$H]Ro 15–4513 binding to benzodiazepine sites, and in furosemide-induced increases in *tert*-[$^{35}$S]butylbicyclophosphorothionate binding to picrotoxin-sensitive convulsant sites. These observations agree with the known specific properties of these sites on recombinant $\alpha6\beta2/3\gamma2$ receptors. In the presence of GABA concentrations that fail to activate α1 subunit-containing receptors, methyl-6,7-dimethoxy-4-ethyl-β-carboline (30 μM), allopregnanolone (100 nM), and $Zn^{2+}$ (10 μM) are less efficacious in altering *tert*-[$^{35}$S]butylbicyclophosphorothionate binding in the granule cell layer of the $\alpha6^{-/-}$ than $\alpha6^{+/+}$ animals. These data concur with the deficiency of the cerebellar α6 and δ subunit-containing receptors in the $\alpha6^{-/-}$ animals and could also account for the decreased affinity of [$^3$H]muscimol binding to $\alpha6^{-/-}$ cerebellar membranes. Predicted additional alterations in the cerebellar receptors of the mutant mice may explain a surplus of methyl-6,7-dimethoxy-4-ethyl-β-carboline-insensitive receptors in the $\alpha6^{-/-}$ granule cell layer and an increased diazepam-sensitivity in the molecular layer. These changes may be adaptive consequences of altered $GABA_A$ receptor subunit expression patterns in response to the loss of two subunits (α and δ) from granule cells.

Receptors responsible for fast inhibitory neurotransmission in the brain, the $GABA_A$ receptors, are extremely heterogeneous. Among the 14 subunits (α1–6, β1–3, γ1–3, δ, and ε) known to participate in forming pentameric mammalian $GABA_A$ receptors (1–3), the α6 subunit displays the most unique features in its cerebellar and cochlear nucleus granule cell-restricted expression (4–6) and benzodiazepine agonist-insensitive pharmacology (7, 8). Furthermore, the α6 subunit imparts high GABA sensitivity (9) and selective furosemide sensitivity (10) to the $GABA_A$ receptors of synapses between the GABAergic inhibitory Golgi neurons and glutamatergic excitatory granule cells (11). These features, also detectable in cultured cerebellar granule cells (12, 13), have made the α6 subunit-containing $GABA_A$ receptors the most distinctive among the $\alpha X\beta2/3\gamma2/\delta$ $GABA_A$ receptors.

Two "α6 knockout" mouse lines have been produced by disrupting the α6 subunit gene through homologous recombination techniques (14, 15). Mutant mice are grossly normal in motor skills and their cerebellar cortical cytoarchitecture, indicating that the α6 subunit is dispensable. However, the homozygous $\alpha6^{-/-}$ animals lack the cerebellar granule cell layer diazepam-insensitive Ro 15–4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate) binding (14, 15), as predicted from earlier work (7, 8, 16). In the experiments by Jones *et al.* (14), a specific association between α6 and δ subunits was revealed; the δ subunit protein was largely absent from the cerebella of $\alpha6^{-/-}$ mice, as demonstrated by immunoprecipitation, immunocytochemistry, and immunoblot analysis with distinct δ subunit-specific antibodies. A decrease in the affinity of cerebellar GABA sites was also observed (14, 15).

The conservation of expression pattern and sequence of the α6 subunit gene in fish, birds, rodents, and humans (17, 18) suggests an important function for α6 subunit-containing

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**ABBREVIATIONS:** GABA, γ-aminobutyric acid; DMCM, methyl-6,7-dimethoxy-4-ethyl-β-carboline; TBPS, *t*-butylbicyclophosphorothionate.

$GABA_A$ receptors in the brain. Thus, the lack of any obvious behavioral phenotype of the $\alpha6^{-/-}$ animals (14, 15) might indicate a compensatory rearrangement of $GABA_A$ receptor subunit composition in the mutant cerebellum. Here, we used pharmacological binding techniques to compare $GABA_A$ receptor fingerprints between wild-type $\alpha6^{+/+}$ and mutant $\alpha6^{-/-}$ cerebella. Previously, it has been shown that allosteric modulation of the convulsant binding site labeled with [$^{35}$S]TBPS is an excellent way to probe differences in receptor subunit combinations (9, 19). We examined the modulation of [$^{35}$S]TBPS binding to receptors in cerebellar cortical layers as affected by the endogenous agonist GABA; the GABA antagonists furosemide, SR 95531 [2′-(3′-carboxy-2′,3′-propyl)-3-amino-6-*p*-methoxyphenylpyrazinium bromide], and $Zn^{2+}$ ions; the benzodiazepine site agonist diazepam and inverse agonist DMCM; and the neurosteroid agonist allopregnanolone (5α-pregnan-3α-ol-20-one). The results suggest altered receptor modulations in both the granule cell and molecular layers of the $\alpha6^{-/-}$ mice, consistent with subtle subunit reconfigurations in mutant cerebella.

## Experimental Procedures

**Materials.** [$^{35}$S]TBPS, [$^3$H]Ro 15–4513, and [$^3$H]SR 95531 were purchased from Dupont-New England Nuclear (Dreieich, Germany), and [$^3$H]methylamine muscimol was purchased from Amersham (Buckinghamshire, UK). Flumazenil (Ro 15–1788) was donated by F. Hoffmann-La Roche (Basel, Switzerland), diazepam was donated by Orion Pharmaceutica (Espoo, Finland), and zolpidem was donated by Synthelabo Recherché (Bagneux, France). GABA, picrotoxinin, and furosemide were purchased from Sigma Chemical (St. Louis, MO). Unlabeled SR 95531, 5α-pregnan-3α-ol-20-one (allopregnanolone), and DMCM were obtained from Research Biochemicals (Natick, MA), and $ZnCl_2$ was from Merck (Darmstadt, Germany).

**Animals.** Two independent 129Sv × C57BL/6 mouse lines, in which the exon 8 of the mouse α6 subunit gene was disrupted at the same site, were created by homologous recombination (14, 15). The brains of 33 homozygous wild-type ($\alpha6^{+/+}$), 33 homozygous mutant ($\alpha6^{-/-}$), and six heterozygous mutant ($\alpha6^{+/-}$) adult (~4-month-old) mice of the $F_2$ and $F_3$ generations were used. Three brains of each genotype for the autoradiography were obtained from the Pittsburgh mice (15), whereas all the other samples originated from the Cambridge line (14).

**Preparation of brain membranes and cryostat sections.** All mice were killed by decapitation, and the whole brains or cerebella were rapidly dissected and frozen on dry ice. For ligand autoradiography, 14-μM horizontal, coronal, and sagittal serial sections were cut from 13 $\alpha6^{+/+}$, 13 $\alpha6^{-/-}$, and six $\alpha6^{+/-}$ brains using a Leitz 1720 cryostat, thaw-mounted onto gelatin-coated object glasses, and stored frozen under desiccant at −20°. To prepare cerebellar membranes, $\alpha6^{+/+}$ and $\alpha6^{-/-}$ cerebella (from the Cambridge line) were weighed and thawed, and membranes were prepared from them for ligand binding assays as previously described in detail (10). Four membrane pools were prepared from both lines with five cerebella in each. All experiments were carried out in parallel fashion in respect to mouse lines, eliminating any day-to-day variation in receptor assays between the lines.

**Ligand autoradiography.** The autoradiographic procedures for regional localization of [$^3$H]Ro 15–4513, [$^3$H]muscimol, [$^3$H]SR 95531, and [$^{35}$S]TBPS binding were as previously described in detail (10, 19–21). Briefly, 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 [$^{35}$S]TBPS and [$^3$H]Ro 15–4513 autoradiographic assays and in 0.31 M Tris-citrate, pH 7.1, in [$^3$H]muscimol and [$^3$H]SR 95531 assays. In some assays, the endogenous GABA, which could interfere with determination of α6 subunit pharmacology (9), was removed by preincubating the sections three times in an ice-water bath for 10 min in 50 mM Tris·HCl supplemented with 1 mM EDTA, pH 7.4.

Final incubations in the preincubation buffer were performed with 6 nM [$^{35}$S]TBPS at room temperature for 90 min, with 6 nM [$^3$H]muscimol and 20 nM [$^3$H]SR 95531 at 0–4° for 30 min, and with 5 nM [$^3$H]Ro 15–4513 at 0–4° for 60 min. The effects of furosemide, SR 95531, $ZnCl_2$, diazepam, DMCM, and allopregnanolone in the presence or absence of 0.5, 1, 3, or 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 and 100 μM zolpidem. After the incubation, sections were washed three times for 15 sec or twice for 30 sec in an ice-cold incubation buffer in [$^{35}$S]TBPS and [$^3$H]Ro 15–4513 or in [$^3$H]muscimol and [$^3$H]SR 95531 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] standards to Hyperfilm-$^3$H or Hyperfilm-$\beta_{max}$ (Amersham), respectively, for 1–6 weeks. Nonspecific binding was determined with 10 μM flumazenil (Ro 15–1788), 10 μM picrotoxinin, and 100 μM GABA in [$^3$H]Ro 15–4513, [$^{35}$S]TBPS, and [$^3$H]muscimol and [$^3$H]SR 95531 assays, respectively. Substantial background binding was obtained only with [$^3$H]SR 95531 (Fig. 1), due to its binding to monoamine oxidase A in a GABA-insensitive manner (22). Images from representative autoradiography films were produced by scanning the films using Arcus II scanner (Agfa Gevaert, Leverkusen, Germany) and Adobe Photoshop (version 3.0; Adobe Systems, Mountain View, CA) program.

**[$^3$H]Muscimol binding assay.** Binding of [$^3$H]muscimol at 10 different concentrations (1–300 nM) was performed in triplicate in a total volume of 250 μl containing ~50–100 μg of cerebellar membrane protein. Incubations were performed for 60 min at 0–4° in 50 mM Tris-citrate buffer, pH 7.1. Nonspecific binding was determined with 100 μM GABA. Incubation was ended by centrifugation at 13,000 × *g* for 10 min at 0–4°. The pellets were rinsed once with 1 ml of ice-cold water, and the bottom of the tube containing the pellet was cut into a scintillation vial. The pellets were dissolved in 0.5 ml of LUMA Solve (Lumac LSC, Groningen, The Netherlands) overnight at room temperature, after which 4 ml of Optiphase HiSafe 2 scintillation fluid (Wallac, Turku, Finland) was added, and radioactivity was determined in a Wallac model 1410 liquid scintillation counter.

**Data analysis.** Autoradiography films were quantified using MCID M4 image analysis devices and programs (Imaging Research, St. Catharines, Ontario, Canada) as described in detail by Korpi *et al.* (19). Binding densities for each brain area were averaged from measurements from 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 $^3$H and nCi/g for $^{14}$C).

Saturation isotherms of [$^3$H]muscimol binding were analyzed for the estimation of $K_d$ and $B_{max}$ by nonlinear regression with Prism 2.0 (GraphPAD Software, San Diego, CA).

Statistical significances of the differences between the $\alpha6^{+/+}$, $\alpha6^{+/-}$, and $\alpha6^{-/-}$ mice groups and between two population means were assessed with Prism by using one-way analysis of variance followed by Newman-Keuls *post hoc* test or by using Student's *t* test, respectively.

## Results

**Benzodiazepine agonist-insensitive binding is absent in the mutant $\alpha6^{-/-}$ mice.** Total [$^3$H]Ro 15–4513 binding was widespread (14, 15), as expected due to the high affinity of [$^3$H]Ro 15–4513 to all $GABA_A$ receptors with benzodiazepine sites (23), and totally displaceable by the benzodiazepine site antagonist flumazenil (Ro 15–1788; data not

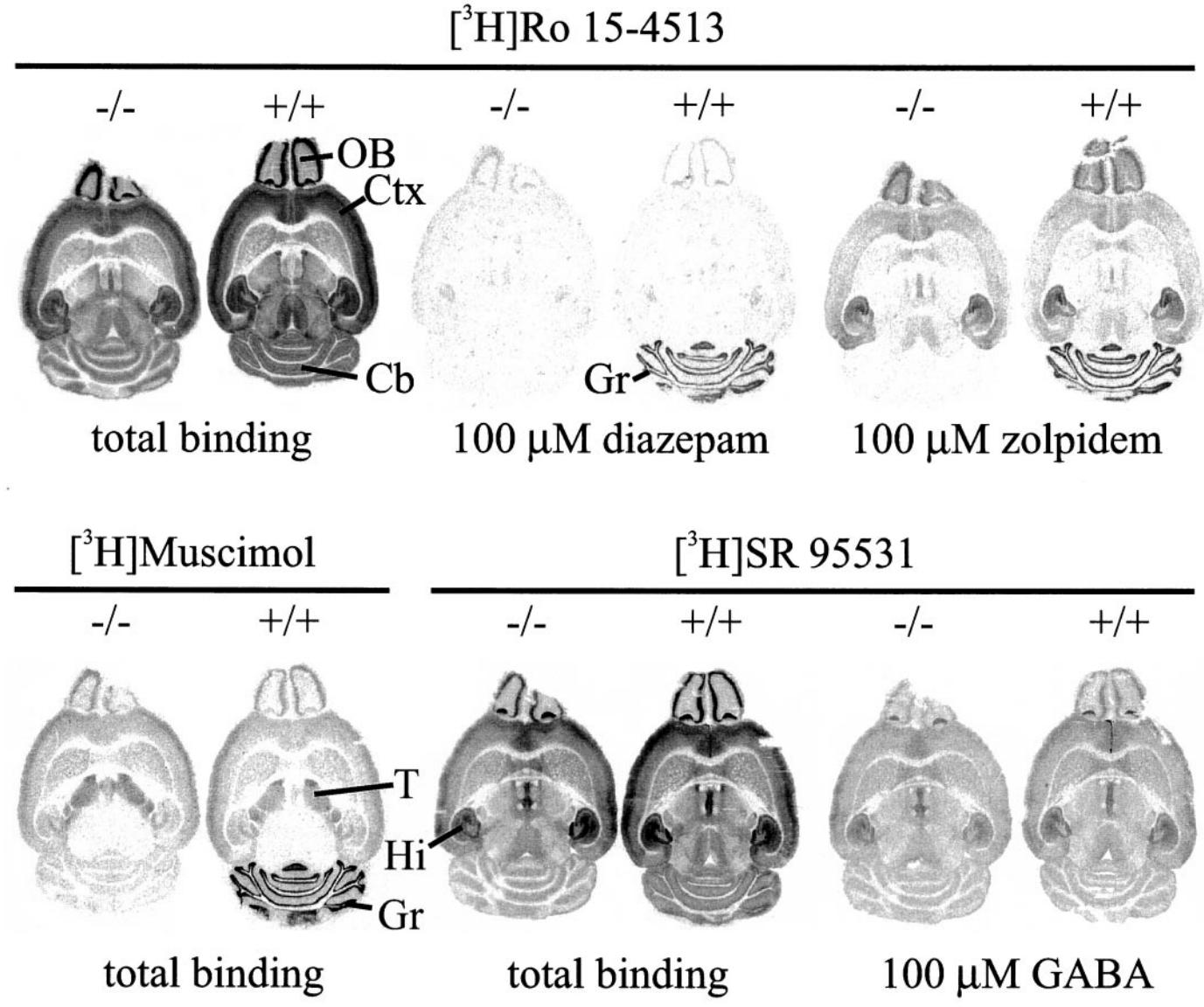


**Fig. 1.** Autoradiographic distribution of $GABA_A$ receptor binding sites in $\alpha6^{-/-}$ and wild-type $\alpha6^{+/+}$ mice. Flumazenil-sensitive benzodiazepine sites were labeled by 5 nM [$^3$H]Ro 15–4513, showing total binding, diazepam-insensitive binding, and zolpidem-insensitive binding. GABA sites were labeled by 6 nM [$^3$H]muscimol, showing total binding, with nonspecific binding in the presence of 100 $\mu$M GABA at the film background level. GABA sites were also labeled by 20 nM [$^3$H]SR 95531, showing total binding and nonspecific binding in the presence of 100 $\mu$M GABA. *OB*, olfactory bulb; *Ctx*, cerebral cortex; *Cb*, cerebellum; *Gr*, cerebellar granule cell layer; *T*, thalamus; *Hi*, hippocampus.

shown) throughout the $\alpha6^{+/+}$, $\alpha6^{+/-}$, and $\alpha6^{-/-}$ brains (Fig. 1). However, there was significantly less [$^3$H]Ro 15–4513 binding to the cerebellar granule cell layer of $\alpha6^{-/-}$ and $\alpha6^{+/-}$ mice compared with binding to that of $\alpha6^{+/+}$ mice (Table 1).

Diazepam-insensitive [$^3$H]Ro 15–4513 binding is considered to be the hallmark of $\alpha6$ subunit-containing $GABA_A$ receptors (4, 7, 8, 16). Diazepam (100 $\mu$M) only partially displaced [$^3$H]Ro 15–4513 binding from the cerebellar granule cell layer of the $\alpha6^{+/+}$ and $\alpha6^{+/-}$ mice, whereas the binding from granule cell layer of $\alpha6^{-/-}$ mice was totally displaceable (Table 1), as also demonstrated by Jones *et al.* (14) and Homanics *et al.* (15). There was very little diazepam-insensitive binding left in the molecular layer of each mouse genotype (Table 1). The diazepam-insensitive binding in the granule cells is also insensitive to the subtype-selective agonist zolpidem (21), which was confirmed in the $\alpha6^{+/+}$ and $\alpha6^{+/-}$ mice.

**GABA site labeling is reduced in cerebellar granule cell layer of the $\alpha6^{-/-}$ mice.** GABA site agonist [$^3$H]muscimol (24) and antagonist [$^3$H]SR 95531 (25) were used as radioligands to determine the regional distribution by autoradiography of GABA binding sites in adult $\alpha6^{+/+}$, $\alpha6^{+/-}$, and $\alpha6^{-/-}$ mice. Both [$^3$H]muscimol and [$^3$H]SR 95531 binding was almost completely missing from cerebellar granule cells of $\alpha6^{-/-}$ mice (Fig. 1; Table 2). Identical images were obtained in $\alpha6^{-/-}$ mouse brains from both sources. The amount of [$^3$H]muscimol binding to the cerebellar granule cell layer of $\alpha6^{+/-}$ mice was not significantly reduced compared with that of the $\alpha6^{+/+}$ mice. The GABA site labeling was thus decreased in $\alpha6^{-/-}$ mice, similar to the benzodiazepine agonist-insensitive site labeling (Table 2). The amount

TABLE 1

**Benzodiazepine-insensitive [$^3$H]Ro 15-4513 binding in cerebellar cortical layers of $\alpha6^{+/+}$, $\alpha6^{+/-}$, and $\alpha6^{-/-}$ mice**

Serial brain sections were incubated with 5 nM [$^3$H]Ro 15-4513 in the presence or absence of diazepam or zolpidem at 100 $\mu$M. Nonspecific binding was defined in the presence of 10 $\mu$M flumazenil. Autoradiographic films were processed and quantified against radioactivity standards. Data are mean ± standard deviation for six animals in each group, with half of each group from the Cambridge line and half from the Pittsburgh line.

| Brain region | Total binding | Diazepam | Zolpidem |
|---|---|---|---|
| | *nCi/mg* | *% of total binding* | |
| Granule cell layer | | | |
| $\alpha6^{+/+}$ | 8.1 ± 1.0 | 62.9 ± 11.2 | 73.5 ± 13.0 |
| $\alpha6^{+/-}$ | 6.4 ± 1.0[a] | 56.7 ± 7.3 | 62.5 ± 9.3 |
| $\alpha6^{-/-}$ | 5.2 ± 0.7[b] | 4.5 ± 1.3[b] | 5.7 ± 2.5[b] |
| Molecular layer | | | |
| $\alpha6^{+/+}$ | 9.7 ± 1.5 | 4.4 ± 1.5 | 4.5 ± 1.2 |
| $\alpha6^{+/-}$ | 9.4 ± 1.5 | 4.5 ± 1.8 | 5.1 ± 1.6 |
| $\alpha6^{-/-}$ | 8.3 ± 1.2 | 2.3 ± 0.5[a] | 3.4 ± 1.8 |

[a] $p < 0.05$, [b] $p < 0.001$, statistical significance of the difference from the wild-type $\alpha6^{+/+}$ values (Newman-Keuls test).

TABLE 2

**The binding of [$^3$H]muscimol, [$^3$H]SR 95531, and [$^{35}$S]TBPS in cerebellar cortical layers of $\alpha6^{+/+}$, $\alpha6^{+/-}$, and $\alpha6^{-/-}$ mice as revealed by quantitative autoradiography**

Absorbance values of the autoradiographic films in relation to radioactivity standards are mean ± standard deviation for six animals in [$^3$H]muscimol (6 mM) and [$^{35}$S]TBPS (6 nM) and for three animals in [$^3$H]SR 95531 (20 nM) experiments in each group, with half of the [$^3$H]muscimol and [$^{35}$S]TBPS groups from the Cambridge line and half from the Pittsburgh line.

| Brain region | [$^3$H]Muscimol | [$^3$H]SR 95531 | [$^{35}$S]TBPS |
|---|---|---|---|
| | *nCi/mg* | | *nCi/g* |
| Granule cell layer | | | |
| $\alpha6^{+/+}$ | 4.23 ± 0.56 | 15.7 ± 0.7 | 227 ± 48 |
| $\alpha6^{+/-}$ | 3.73 ± 0.75 | N.D. | 217 ± 38 |
| $\alpha6^{-/-}$ | 0.47 ± 0.21[b] | 2.0 ± 0.3[b] | 186 ± 49 |
| Molecular layer | | | |
| $\alpha6^{+/+}$ | 0.79 ± 0.09 | 4.8 ± 1.7 | 148 ± 52 |
| $\alpha6^{+/-}$ | 0.71 ± 0.14 | N.D. | 148 ± 24 |
| $\alpha6^{-/-}$ | 0.57 ± 0.11[a] | 3.4 ± 0.5 | 116 ± 37 |

[a] $p < 0.05$, [b] $p < 0.001$, Statistical significance of the difference from the wild-type $\alpha6^{+/+}$ values (Newman-Keuls or Student's *t* test).
N.D., not determined.

of [$^3$H]muscimol binding to the cerebellar molecular layer of $\alpha6^{-/-}$ mice was slightly ($p < 0.05$) lower than that to the molecular layer of $\alpha6^{+/+}$ mice.

Because the autoradiographic signal of [$^3$H]muscimol picks up only a subpopulation of all cerebellar GABA$_A$ receptors (see Ref. 20), possibly corresponding to $\alpha6\delta$ variants (14), we extended the analysis by running saturation isotherms of [$^3$H]muscimol binding to cerebellar membranes using a centrifugation assay. The binding values fitted a one-component model, as is evident from the Scatchard transformations shown in Fig. 2. The results indicated a significantly lower affinity ($K_d$ increased to 176% of the wild-type value, $p < 0.01$) and maximal density ($B_{max}$ reduced to 55% of the wild-type value, $p < 0.05$) in the $\alpha6^{-/-}$ mice (Fig. 2), respectively. This may explain the low granule cell layer labeling by 6 nM (and 20 nM; not shown) [$^3$H]muscimol in $\alpha6^{-/-}$ mice under autoradiographic conditions and might correlate with the loss of $\alpha6$ and $\delta$ subunit-containing receptors (26).

**Selective lack of furosemide actions in the $\alpha6^{-/-}$ mice.** Furosemide is a loop diuretic that also acts as a selective, noncompetitive antagonist for cerebellar granule cell-specific $\alpha6$ and $\beta2$ or $\beta3$ subunit-containing GABA$_A$ receptors (10). In contrast, SR 95531 has been shown to antagonize most GABA$_A$ receptor populations throughout the brain (19). As expected, both furosemide (200 $\mu$M) and SR 95531 (10 $\mu$M) increased the basal [$^{35}$S]TBPS binding in the absence of exogenous GABA to the granule cell layer of $\alpha6^{+/+}$ mice cerebella (253 ± 33% and 181 ± 24% of basal values, mean ± standard deviation, 10 animals, respectively) (Fig. 3), whereas in $\alpha6^{-/-}$ granule cells, furosemide did not affect binding (98 ± 10% of basal binding), and SR 95531 reduced binding (63 ± 10% of basal binding). In the cerebellar molecular layer of both $\alpha6^{+/+}$ and $\alpha6^{-/-}$ mice, furosemide did not affect binding, and SR 95531 tended to decrease basal [$^{35}$S]TBPS binding (Fig. 3).

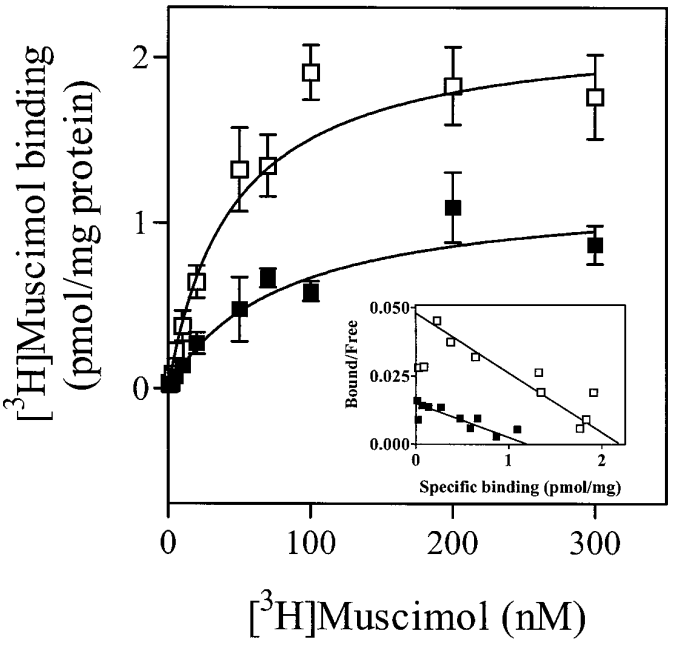


**Fig. 2.** Saturation analysis of [$^3$H]muscimol binding to cerebellar membranes of $\alpha6^{-/-}$ (■) and wild-type $\alpha6^{+/+}$ (□) mice. Membranes were incubated with various concentrations of [$^3$H]muscimol (1–300 nM). Nonspecific binding was determined in the presence of 100 $\mu$M GABA. The specific binding values are mean ± standard error for independent experiments on four $\alpha6^{-/-}$ and four $\alpha6^{+/+}$ membrane preparations. Binding data were analyzed as described in Experimental Procedures. $K_d$ values were 80.5 ± 13.9 versus 45.7 ± 11.9 nM (mean ± standard deviation, four measurements), and $B_{max}$ values were 1.2 ± 0.3 versus 2.2 ± 0.6 pmol/mg of protein for the $\alpha6^{-/-}$ and $\alpha6^{+/+}$ mice, respectively. *Inset*, Scatchard plots of the same data illustrating the decreased affinity and maximal binding site density in the $\alpha6^{-/-}$ membranes.

Exogenous GABA (5 $\mu$M) affected [$^{35}$S]TBPS binding similarly in the granule cell and molecular layers of both $\alpha6^{+/+}$ and $\alpha6^{-/-}$ mice (24 ± 2% and 19 ± 7% compared with 24 ± 6% and 20 ± 7% of basal binding, respectively). Furosemide (200 $\mu$M) was able to reverse inhibition by GABA and elevate the binding over the basal level (145 ± 14% of basal) selectively in the granule cell layer of $\alpha6^{+/+}$ mice cerebella, which is in agreement with studies on Wistar rats (10). Furosemide had no effect on $\alpha6^{-/-}$ cerebella. On the other hand, 10 $\mu$M SR 95531 was able to antagonize the GABA inhibition of [$^{35}$S]TBPS binding in the granule cells of $\alpha6^{+/+}$ mice (139 ± 23% of basal) and in those of $\alpha6^{-/-}$ animals (76 ± 14% of basal). SR 95531 reversed the GABA inhibition of [$^{35}$S]TBPS binding of the molecular layer in a qualitatively similar manner in both $\alpha6^{+/+}$ and $\alpha6^{-/-}$ mice (Fig. 3). These data are in agreement with complete disappearance of $\alpha6$ subunit-containing GABA$_A$ receptors.

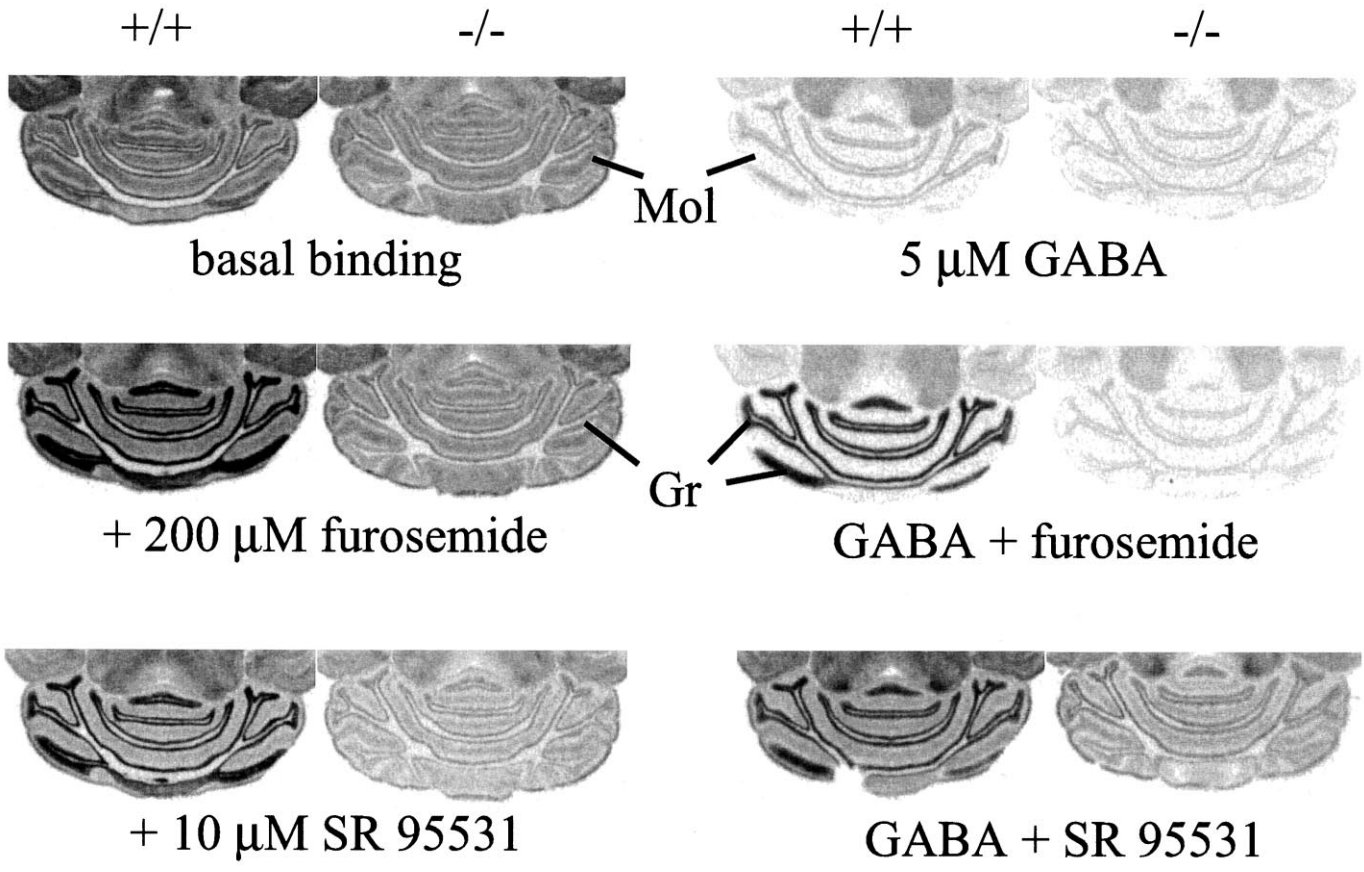


**Fig. 3.** GABA antagonistic actions of furosemide and SR 95531 on [$^{35}$S]TBPS binding in $\alpha6^{-/-}$ and wild-type $\alpha6^{+/+}$ mouse cerebellar sections. Representative autoradiographs of picrotoxinin-sensitive [$^{35}$S]TBPS binding in serial $\alpha6^{-/-}$ and $\alpha6^{+/+}$ sections show basal binding, binding in the presence of 200 $\mu$M furosemide, 10 $\mu$M SR 95531, 5 $\mu$M GABA, GABA plus furosemide, and GABA plus SR 95531. Note the selective lack of action of furosemide and the lack of granule cell layer (*Gr*) antagonism by SR 95531 in the $\alpha6^{-/-}$ mice. *Mol*, molecular layer.

**Decreased GABA antagonism by zinc in the $\alpha6^{-/-}$ mice.** Recombinant $GABA_A$ receptor isoforms have been shown to display differential sensitivity to antagonism by the divalent cation $Zn^{2+}$ (27), with GABA currents of $\alpha6\beta3\delta$ receptors being the most sensitive to inhibition by $Zn^{2+}$ (28). $Zn^{2+}$ (10 $\mu$M) elevated basal [$^{35}$S]TBPS binding in the granule cell layer of $\alpha6^{+/+}$ mice but slightly decreased the binding in the $\alpha6^{-/-}$ mice (Fig. 4). This indicates that the ability of $Zn^{2+}$ ions to antagonize endogenous GABA is diminished in the mutant mice, which is consistent with the lack of $\alpha6\delta$-containing receptors. $Zn^{2+}$ (10 $\mu$M) did not significantly affect [$^{35}$S]TBPS binding to molecular layer (Fig. 4) or to forebrain areas in either mouse line (data not shown). Both 100 and 300 $\mu$M $Zn^{2+}$ reduced [$^{35}$S]TBPS binding to cerebellar granule cell and molecular layers and to forebrain regions of $\alpha6^{+/+}$ and $\alpha6^{-/-}$ mice. Agonism/antagonism of various compounds at the $GABA_A$ receptor can be predicted by the convulsant binding assay only in the presence of a relevant GABA concentration (see Ref. 19); therefore, the differential inhibition of the [$^{35}$S]TBPS binding by higher $Zn^{2+}$ concentrations (Fig. 4) (see Ref. 29) may be functionally meaningless and just represent another allosteric action of $Zn^{2+}$, in the same way as other $GABA_A$ antagonists do in the absence of GABA (19). However, this $Zn^{2+}$ inhibition of the convulsant binding to the cerebellar granule cell layer was significantly greater in $\alpha6^{-/-}$ mice than in $\alpha6^{+/+}$ mice, indicating the presence of different $GABA_A$ receptor populations in the $\alpha6^{-/-}$ and $\alpha6^{+/+}$ granule cells.

**Reduced allopregnanolone sensitivity in the $\alpha6^{-/-}$ mice.** The expression of the $\delta$ subunit in recombinant receptors together with $\alpha1/6\beta3$ or $\alpha1/6\beta3\gamma2$ subunit combinations has been shown to reduce neurosteroid-induced potentiation of GABA-activated currents (13). To determine the effect of $\alpha6$ subunit, we used a longer preincubation time for the sections (see Experimental Procedures) and an incubation solution with a lower GABA concentration (0.5 $\mu$M). Under these conditions, the neurosteroid agonist allopregnanolone (100 nM) decreased ($p < 0.01$) the binding to 75 ± 13% (mean ± standard deviation, three animals) of the values in the presence of GABA alone (an increase in GABA action) in the granule cell layer of $\alpha6^{+/+}$ but was ineffective (97 ± 9%) in $\alpha6^{-/-}$ mice. Allopregnanolone at 10 $\mu$M reduced [$^{35}$S]TBPS binding in all brain regions to the background level in both mouse lines and under both preincubation conditions (data not shown). These results are consistent with the presence of $\alpha1\beta X\gamma2$ and absence of highly GABA-sensitive $\alpha6\delta$ receptors in the $\alpha6^{-/-}$ granule cells.

**Unexpected interaction of diazepam with cerebellar $GABA_A$ receptors of the $\alpha6^{-/-}$ mice.** Diazepam (1 and 30 $\mu$M) produced a greater enhancement of the [$^{35}$S]TBPS binding inhibition by 0.5 $\mu$M GABA in the molecular layer of the $\alpha6^{-/-}$ than $\alpha6^{+/+}$ mice (Table 3; Fig. 5), suggesting that the Golgi/granule cell synapses are not the only altered loci in the mutant mice. Diazepam was less efficacious in the granule cell layer than in the molecular layer in both wild-type and mutant mice.

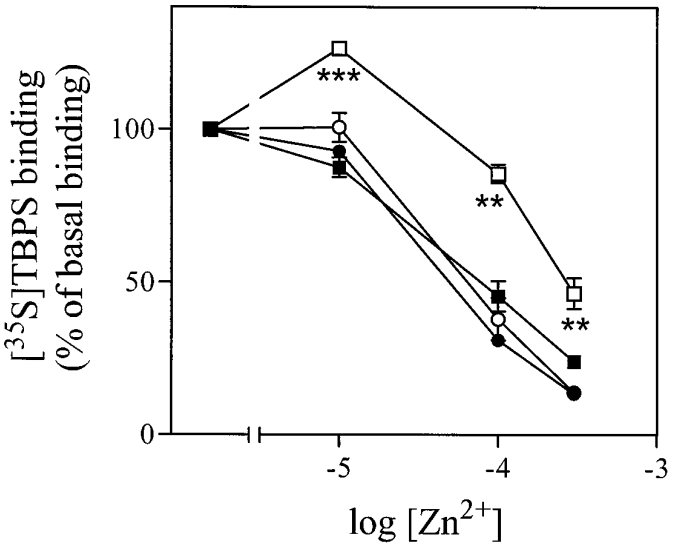


**Fig. 4.** Effect of $Zn^{2+}$ on the picrotoxinin-sensitive [$^{35}$S]TBPS binding in the cerebellar granule cell (■, □) and molecular (●, ○) layers of $\alpha6^{-/-}$ (■, ●) and wild-type $\alpha6^{+/+}$ (□, ○) mouse brain sections. The autoradiographic results are expressed as percentages (mean ± standard error of three measurements) of basal [$^{35}$S]TBPS binding (100%). *Values to the left of the gap*, obtained in the absence of $Zn^{2+}$. **, $p < 0.01$; ***, $p < 0.001$, statistical significance of the difference from the corresponding value of the $\alpha6^{-/-}$ mice (Student's *t* test).

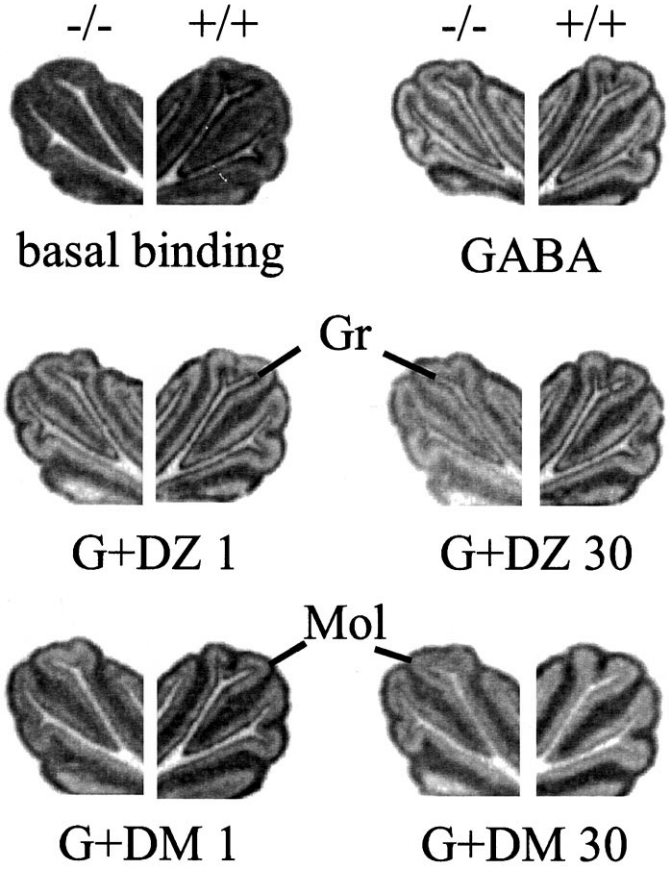


**Fig. 5.** Actions of diazepam (*DZ*) and DMCM (*DM*) in the presence of a low GABA (*G*) concentration (0.5 μM) on picrotoxinin-sensitive [$^{35}$S]TBPS binding in serial $\alpha6^{-/-}$ and wild-type $\alpha6^{+/+}$ mouse cerebellar sections as revealed by autoradiography. Brain sections were washed extensively before incubation to remove endogenous GABA as described in Experimental Procedures. Concentrations of diazepam and DMCM are given in μM. *Gr*, granule cell layer; *Mol*, molecular layer.

**Agonistic modulation by DMCM is reduced in the $\alpha6^{-/-}$ mice.** The β-carboline benzodiazepine-site ligand DMCM acts as an inverse agonist at low micromolar concentrations and as an "agonist" through the loreclezole binding site of the β2 or β3 subunits (30) at higher micromolar concentrations (31). This potentiation is independent of the α subunit and is more pronounced on α6 subunit-containing receptors due to the lack of DMCM inhibition (inverse agonism) via the benzodiazepine site (28, 31). With a thorough preincubation, 30 μM DMCM potentiated the effect of 0.5 μM GABA on [$^{35}$S]TBPS binding significantly ($p < 0.01$) more in the granule cell layer of $\alpha6^{+/+}$ than of $\alpha6^{-/-}$ mice (Fig. 5, Table 3), whereas no such difference was observed in the molecular layer (Table 3).

The benzodiazepine antagonist flumazenil blocked the inverse agonist action of DMCM in the molecular layer, indicating that this action was mediated by the benzodiazepine site (Fig. 6). Flumazenil failed to antagonize the agonistic effect of DMCM in the granule cell layer. The differential action of 30 μM DMCM between the $\alpha6^{+/+}$ and $\alpha6^{-/-}$ mice was observed in the presence of a low (0.5 μM) but not of a high (3 μM) concentration of GABA. Thus, the DMCM actions indicate that the mutant granule cell layer has an increased density of sites with low sensitivity to GABA and/or to "agonistic" action of DMCM through the loreclezole site.

## Discussion

By comparing $\alpha6^{-/-}$ mouse lines with wild-type mouse lines, we directly confirmed the specific pharmacological features of the cerebellar granule cell layer generated by α6 subunit-containing GABA$_A$ receptors. First, the diazepam-

TABLE 3

**Effects of GABA, diazepam, and DMCM on [$^{35}$S]TBPS binding in cerebellar granule cell and molecular layers of $\alpha6^{-/-}$ and $\alpha6^{+/+}$ mice**

The statistical significance of the differences from the basal binding in granule cell layer and molecular layer within the $\alpha6^{+/+}$ and $\alpha6^{-/-}$ mice groups was determined using one-way analysis of variance followed by Newman-Keuls multiple-comparison test. Data are mean ± standard deviation for three animals in each group.

| Drug | Granule cell layer | | Molecular layer | |
|---|---|---|---|---|
| | $\alpha6^{+/+}$ | $\alpha6^{-/-}$ | $\alpha6^{+/+}$ | $\alpha6^{-/-}$ |
| | *nCi/g* | | | |
| Basal binding | 249 ± 42 | 206 ± 8 | 297 ± 28 | 241 ± 11 |
| GABA, 0.5 μM | 185 ± 20 | 174 ± 27 | 259 ± 27 | 207 ± 50 |
| + Diazepam, 1 μM | 197 ± 35 | 182 ± 19 | 239 ± 49 | 156 ± 17[a] |
| + Diazepam, 30 μM | 169 ± 3 | 149 ± 27 | 205 ± 27 | 128 ± 39[b] |
| + DMCM, 1 μM | 193 ± 49 | 168 ± 24 | 269 ± 50 | 201 ± 22 |
| + DMCM, 30 μM | 109 ± 11[a] | 140 ± 22[a] | 233 ± 35 | 195 ± 19 |

[a] $p < 0.05$, [b] $p < 0.01$.

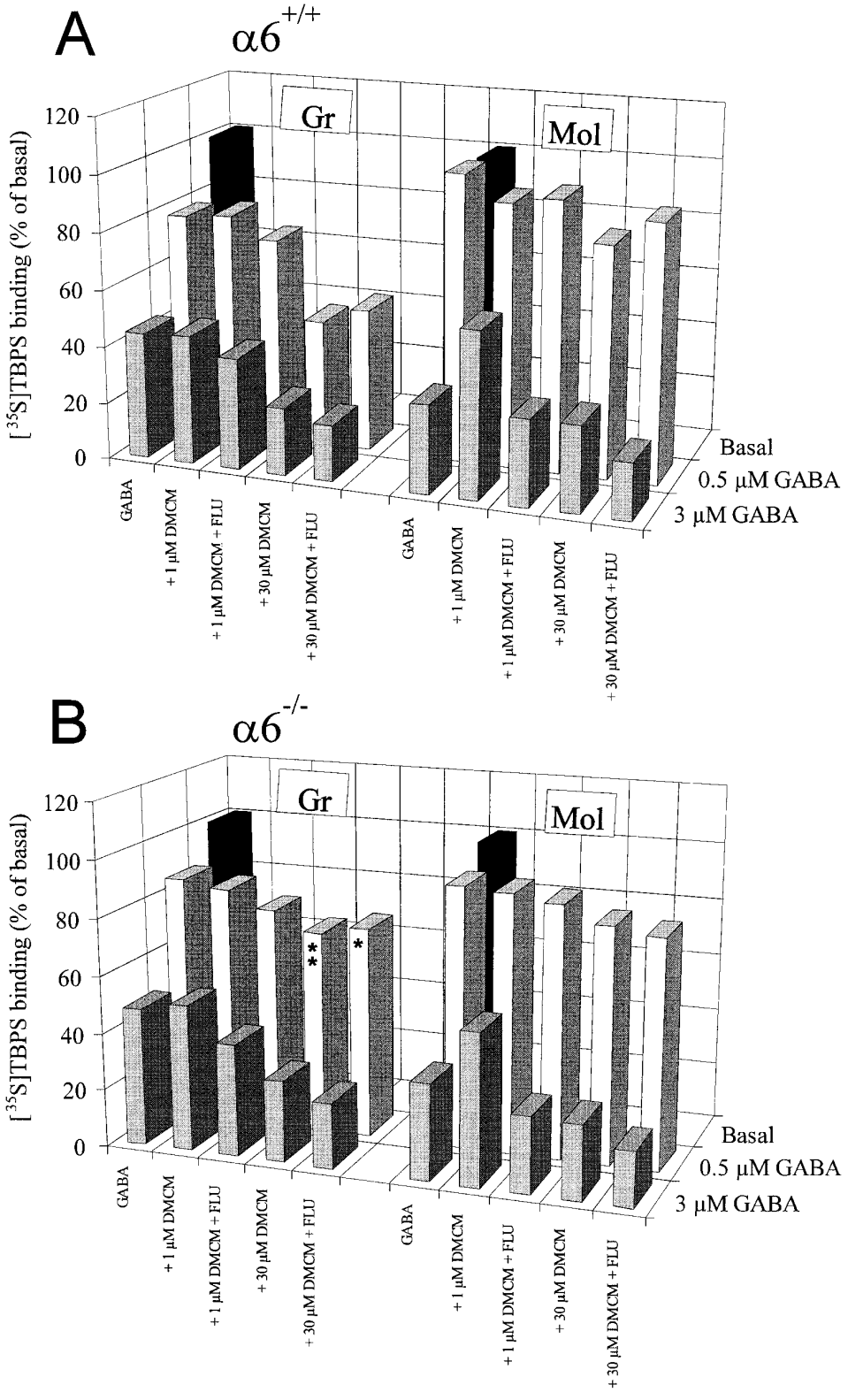


**Fig. 6.** Effect of the benzodiazepine antagonist flumazenil (*FLU*) on the action of DMCM in the cerebellar granule cell (*Gr*) and molecular (*Mol*) layers of wild-type $\alpha6^{+/+}$ (A) and $\alpha6^{-/-}$ (B) mice. Data are mean values (three measurements) with standard deviations (not shown) within 25% of the mean values and data demonstrate a flumazenil-insensitive "agonistic" action of 30 $\mu$M DMCM in the granule cell layer of $\alpha6^{+/+}$ mice at low 0.5 $\mu$M GABA, action that is absent from the mutant mice. $* p < 0.05$; $** p < 0.01$, statistical significance of the difference from the wild-type values (Student's *t* test).

insensitive [$^3$H]Ro 15–4513 binding (7, 8), assumed to represent the benzodiazepine site of $\alpha6\beta2/3\gamma2$ receptors (4) due to diazepam-sensitive $\alpha1$, $\alpha2$, $\alpha3$, and $\alpha5$ subunits (each with histidine as the 100th residue) being replaced with the $\alpha6$ subunit containing Arg100 (32), was absent in the granule cell layer of $\alpha6^{-/-}$ mice. Second, the high GABA sensitivity of cerebellar granule cell receptors and recombinant $\alpha6\beta2/3\gamma2$ receptors (9, 28, 33) was confirmed; the $\alpha6^{-/-}$ mice exhibited the lack of increase in convulsant ([$^{35}$S]TBPS) binding in the presence of the GABA site antagonist SR 95531. Third, selective furosemide antagonism of GABA-induced inhibition of [$^{35}$S]TBPS binding in the cerebellar granule cell layer and recombinant $\alpha6$ subunit-containing receptors (10) was absent in the $\alpha6^{-/-}$ mice.

The δ subunit protein is largely lost from the cerebellar granule neurons of the $\alpha6^{-/-}$ mice (14). The α6 subunit-containing receptors are more sensitive to GABA and $Zn^{2+}$ than α1-containing receptors (9, 28, 33), and both of these properties are further accentuated by the δ subunit: δ subunit-containing receptors have an extremely high affinity to GABA agonists and a great sensitivity to functional antagonism by $Zn^{2+}$ (28). These properties were lost in the granule cell layer of $\alpha6^{-/-}$ mice (diminished [$^3$H]muscimol binding and lower GABA-antagonism by $Zn^{2+}$). These results suggest that the loss of δ subunit in the cerebellar granule cell layer of the $\alpha6^{-/-}$ mice, producing a brain-region specific double-subunit inactivation, is clearly detectable pharmacologically. The δ subunit-containing receptors have reduced sensitivity to neurosteroid agonists (13), but we observed no enhancement by allopregnanolone of the action of 0.5 μM GABA in the mutant δ-deficient granule cell layer. This might be explained by the requirements by δ-deficient receptors of higher GABA concentrations to reveal the allosteric effects of the neurosteroid agonist.

In addition to these gross pharmacological alterations, a few subtle novel properties have emerged in cerebellar $GABA_A$ receptors of the $\alpha6^{-/-}$ mice. To avoid the interference of endogenous GABA with the α6 subunit-containing $GABA_A$ receptors (9), the brain sections were washed extensively before [$^{35}$S]TBPS incubations to reveal allosteric interactions. Using a very low exogenous GABA concentration (0.5 μM), we could dissect the allosteric actions on the α1 and α6 subunit-containing receptors. DMCM acts allosterically at low micromolar concentrations as an inverse agonist but as an "agonist" through another binding site dependent on the β2 and/or β3 subunits (30) at higher micromolar concentrations (31). The latter agonistic action has been shown to be more pronounced on α6 subunit-containing receptors due to the lack of DMCM inhibition (inverse agonism) via the benzodiazepine site (28, 31). Consistent with this, the inverse agonism of DMCM was absent in the granule cell layer of $\alpha6^{+/+}$ and $\alpha6^{-/-}$ mouse strains, but DMCM was more efficacious in the $\alpha6^{+/+}$ mouse strain than in the $\alpha6^{-/-}$ mouse strain at a high "agonistic" concentration of 30 μM (Fig. 6). This finding was particularly notable because the actual amount of [$^{35}$S]TBPS binding remaining unaffected with 30 μM DMCM in the $\alpha6^{-/-}$ cerebellar granule cell layer was greater even in absolute density units than that found in the $\alpha6^{+/+}$ cerebella (Table 3; Fig. 4). This indicates an increase in the density of receptors with reduced DMCM sensitivity in the $\alpha6^{-/-}$ granule cells. In the presence of 3 μM GABA, 30 μM DMCM potentiated the GABA inhibition of the granule cell layer [$^{35}$S]TBPS binding down to ~25% of the basal binding in both mouse genotypes (Fig. 6). Thus, in sections incubated with a low GABA concentration, the agonism by DMCM is α subunit dependent. This is primarily due to the differing GABA sensitivity between $\alpha6\beta2/3\gamma2$ and $\alpha1\beta2/3\gamma2$ receptors. In addition, the inverse agonist action of DMCM is limited on $\alpha6\beta2/3\gamma2$ receptors due to the reduction by Arg100 of the affinity of DMCM (4, 31). The key conclusion is that the $\alpha6^{-/-}$ cerebellar granule cell layer has an increased number of receptors (e.g., $\alpha1\beta2/3\gamma2$ receptors) with such a low GABA sensitivity that 0.5 μM GABA is inefficient to promote the agonistic action of DMCM.

Regardless of the mechanism behind this alteration, it implies that in the absence of α6 and δ subunits, the remaining granule cell subunits assemble into slightly different combinations. This rearrangement could compensate for the loss of inhibition by α6δ-containing receptors in the $\alpha6^{-/-}$ cerebellar granule cell layer. Because the remaining subunits should be α1, β2, β3, and γ2 (Ref. 6), it is difficult to account for the altered pharmacology unless a substantial proportion of the granule cell $GABA_A$ $\alpha1\beta2/3\gamma2$ receptors are normally pharmacologically "masked" in the wild-type animals, perhaps by the presence of a dominant α6 subunit in the same complex. However, this possibility is controversial (6, 9, 34, 35). An alternate explanation might be an increased amount of β1 subunit-containing receptors in the $\alpha6^{-/-}$ cerebella because this β subunit is insensitive to the agonistic action of DMCM due to a single amino acid residue change (Ser290 in β1 versus Asn290 in β2 and β3 subunits; Ref. 31). Although the β1 subunit mRNA is normally rare in cerebellar granule cells (36, 37), the possible increase in the proportion of β1 among the β subunit variant proteins should be explored.

Another unexpected finding was the higher efficacy of diazepam in the cerebellar molecular layer of $\alpha6^{-/-}$ mice in the presence of low GABA concentrations. The molecular layer normally has $\alpha1\beta2/3\gamma2$ receptors on the dendrites of Purkinje and stellate/basket cells (6, 38). At these receptors, diazepam is only a partial agonist (39). Adult Bergman glial cells normally express only α2 and γ1 subunits (6), a combination that should be little affected by diazepam (40). Therefore, the novel pharmacology of the molecular layer of the $\alpha6^{-/-}$ animals predicts the presence of other subunit combinations with enhanced GABA and/or benzodiazepine sensitivity.

In conclusion, our results regarding the $GABA_A$ receptor α6 subunit gene knockout mouse lines confirm the specific pharmacological features of the α6 and δ subunit-containing receptors in the cerebellar granule cell layer. In addition, the data revealed several unexpected alterations in the $\alpha6^{-/-}$ cerebella, which could be explained by subtle compensatory subunit reconfigurations in the cerebellar cortex.

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