Molecular and Pharmacological Characterization of GABA<sub>A</sub> Receptor α1 Subunit Knockout Mice


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ABSTRACT

GABA<sub>A</sub> receptors mediate fast inhibitory neurotransmission in the central nervous system (CNS), and approximately half of these receptors contain α1 subunits. GABA<sub>A</sub> receptor α1 subunits are important for receptor assembly and specific pharmacological responses to benzodiazepines. Plasticity in GABA<sub>A</sub> receptor α1 subunit expression is associated with changes in CNS excitability observed during normal brain development, in animal models of epilepsy, and upon withdrawal from alcohol and benzodiazepines. To examine the role of α1 subunit-containing GABA<sub>A</sub> receptors in vivo, we characterized receptor subunit expression and pharmacological properties in cerebral cortex of knockout mice with a targeted deletion of the α1 subunit. The mice are viable but exhibit an intention tremor. Western blot analysis confirms the complete loss of α1 subunit peptide expression. Stable adaptations in the expression of several GABA<sub>A</sub> receptor subunits are observed in the fifth to seventh generations, including decreased expression of β2/3 and γ2 subunits and increased expression of α2 and α3 subunits. There was no change in α4, α5, or δ subunit peptide levels in cerebral cortex. Knockout mice exhibit loss of over half of GABA<sub>A</sub> receptors measured by [3H]muscimol, [3H]2-(3-carboxy)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide ([3H]Ro15-4513), and t-butylbicyclophosphoro[35S]thionate ([35S]TBPS) binding, [3H]Ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate ([3H]Ro15-4513) binding is reduced by variable amounts in different regions across brain. GABA<sub>A</sub> receptor α1<sup>−/−</sup> mice lose all high-affinity [3H]zolpidem binding and about half of [3H]flunitrazepam binding in the cerebral cortex. The potency and maximal efficacy of muscimol-stimulated 36Cl<sup>−</sup> uptake in cerebral cortical synaptoneurosomes are reduced in α1<sup>−/−</sup> mice. Furthermore, knockout mice exhibit increased bicuculline-induced seizure susceptibility compared with wild-type mice. These data emphasize the significance of α1 subunit expression and its involvement in the regulation of CNS excitability.

GABA<sub>A</sub> receptors are a family of ligand-gated ion channels that are the major target of the endogenous inhibitory neurotransmitter (GABA) and maintain the majority of fast inhibitory ion currents in the CNS. They are pentamers composed of subunits (α1–6, β1–3, γ1–3, δ, ε, θ, and π) that are encoded by a gene family with diverse expression patterns (Sieghart et al., 1999). GABA<sub>A</sub> receptors are the targets of several classes of drugs, including benzodiazepines (BZDs), barbiturates, alcohols, neurosteroids, and inhalation anesthetics (Sieghart, 1995). Additionally, GABA<sub>A</sub> receptors have been shown to be involved in epilepsy (DeLorey et al., 1998), various behavioral states such as depression and anxiety (Benson et al., 1998; Crestani et al., 1999), and learning and memory (Flood et al., 1992; DeLorey et al., 1998).

Prevailing theory suggests that the subunit composition of an individual GABA<sub>A</sub> receptor confers a unique pharmacology that dictates the binding characteristics, functional capacity, and role of the receptor in maintaining the inhibitory tone of the CNS. The GABA<sub>A</sub> receptor α1 subunit is the most abundant α subunit in adult brain, highly expressed throughout most brain regions, and is a component of ~50% of GABA<sub>A</sub> receptors (Duggan and Stephenson, 1990; McKernan et al., 1991). Recombinant expression studies have indicated that α1 subunit expression confers specific pharmacological properties to the receptor, including GABA sensitivity (Levitan et al., 1988) and maximal efficacy of benzodiazepines (Puia et al., 1991). Furthermore, the expression of α1 versus α2, α3, and α5 in αβγ2 receptors results in differential

ABBREVIATIONS: CNS, central nervous system; BZD, benzodiazepine; mIPSC, miniature inhibitory postsynaptic potential; ANOVA, analysis of variance; TBPS, t-butylbicyclophosphoro[35S]thionate; Ro15-4513, [3H]ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; SR-95531, 2-(3-carboxyl)-3-amino-6-(4-methoxyphenyl)-pyridazinium bromide; CL 218, 872, 3-methyl-6-(8-trifluoromethyl-phenyl)-triazolo[4,3-b] pyridazine.
affinity for several benzodiazepine site ligands (Pritchett et al., 1989; Pritchett and Seeburg, 1990).

Recently, several studies have begun to describe the in vivo role of the α1 subunit in GABA<sub>A</sub> receptor pharmacology, function, and related behavior. An association between heightened CNS excitability and reduced α1 subunit expression has been observed during ontogenic development, alcohol dependence and withdrawal, and in animal models of temporal lobe epilepsy (Aicardi and Chevrie, 1970; Mecarelli et al., 1988; Morrow et al., 1990; Devaud et al., 1997; Brooks-Kayal et al., 1998; Poulter et al., 1999). Previous studies have suggested various roles for a subunit isoforms in specific BZD-related behaviors (Mohler et al., 1996; Rudolph et al., 1999; Crestani et al., 2000; Low et al., 2000). Recently, the production of two independent α1 knockout mouse lines has been described (Sur et al., 2001; Vicini et al., 2001). Global deletion of the α1 subunit gene results in viable mice that are surprisingly normal. Although initial electrophysiological studies revealed diminished mIPSCs and loss of zolpidem-induced prolongation of mIPSC decay rates in cerebellar stellate cells (Vicini et al., 2001), we have now analyzed GABA<sub>A</sub> receptor subunit expression, ligand binding, and muscimol-stimulated Cl<sup>-</sup> uptake in cerebral cortex as well as ligand binding autoradiography throughout brain. The results reveal interesting stable receptor adaptations that differ in some respects from adaptations observed in α1<sup>−/−</sup> mice reported by Sur et al. (2001). The goals of the present studies were to identify in vivo relationships between GABA<sub>A</sub> receptor subunit expression, receptor adaptations, function, and seizure susceptibility.

Materials and Methods

Subjects. Male and female wild-type (α1<sup>+/+</sup>), heterozygous GABA<sub>A</sub> receptor α1 subunit knockout (α1<sup>−/−</sup>), and homozygous GABA<sub>A</sub> receptor α1 subunit knockout (α1<sup>−/−</sup>) mice (Vicini et al., 2001) were derived from α1<sup>−/−</sup> breeding pairs at the University of North Carolina (Chapel Hill, NC) or the University of Pittsburgh (Pittsburgh, PA). The wild-type allele consisted of a floxed allele in which the exon encoding nucleotides 1307 to 1509 of the α1 subunit was flanked by loxP sites that lacked a selectable marker gene. The knockout α1 allele consisted of the floxed allele after cre-mediated recombination. Briefly, the floxed allele was produced in Strain 129/Sv/SvJ embryonic stem cells. Chimeric offspring derived from these cells were mated to C57BL/6J mice and subsequently interbred for one generation. These mice were crossed with an actin-cre general deleter mouse line (FVB/N genetic background) to produce the recombined allele (Lewandoski et al., 1997). The cre transgene was subsequently eliminated from the pedigree. Mice that were heterozygous for the wild-type floxed allele and the recombined allele were interbred to produce the mice for experimental analysis. Thus, all mice were of the same mixed genetic background consisting of C57BL/6J (−37.5%), 129/Sv/SvJ (−37.5%), and FVB/N (−25%). All animals were genotyped by Southern blot analysis, as described previously (Vicini et al., 2001). After weaning, mice were group housed with same sex littermates, given free access to standard rodent chow and water, and maintained on a 12-h alternating light/dark schedule with lights on at 7:00 AM. All studies were conducted with mice derived from F5 to F7 generations and were 8 to 13 weeks of age. All studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by each institution’s Animal Care and Use committees.

Western Blot Analysis. After decapitation, cerebral cortex was rapidly dissected over ice, frozen on dry ice, and stored at −80°C. P2 membrane fractions from cerebral cortex were prepared by homogenization in phosphate-buffered saline buffer (150 mM NaCl and 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4). Aliquots of 25 μg protein/lane were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions using an Xcell II minicell apparatus (Novex, San Diego, CA). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Blots were probed with GABA<sub>A</sub> receptor anti-peptide α1, α2, α3, α5, and γ2 (Fritschy and Mohler, 1995), α4 (Kern and Sieghart, 1994), β2/3 (bd17; BMB, Indianapolis, IN), and δ (Quirk et al., 1995) antibodies. Antibodies were kind gifts from Drs. Jean-Marc Fritschy (University of Zurich, Zurich, Switzerland) and Werner Sieghart (University of Vienna, Vienna, Austria). Blots were then probed with horseradish peroxidase-conjugated anti-guinea pig α1, 2, 3, and 5 and γ2), anti-rabbit α4 and δ), or anti-mouse β2/3, actin antibodies. Specific peptide labeling was detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Blots were exposed to X-ray film (Eastman Kodak, Rochester, NY) under nonsaturating conditions and analyzed by densitometric measurements (NIH Image 1.47). All Western blots were conducted under conditions in which densitometric signals were linear with protein concentration as determined in preliminary experiments. Blots were reprobed with actin and normalized to verify equivalent protein loading.

Radioligand Binding. After decapitation, brains were immediately removed and placed in ice-cold saline from which cerebral cortices were rapidly dissected over ice and either used immediately or frozen on dry ice and stored at −80°C. Membranes were prepared by homogenization of cerebral cortices from eight mice per genotype in 50 volumes of assay buffer (50 mM Tris-citrate, pH 7.4) with a Polytron homogenizer. Samples were centrifuged at 20,000g for 20 min and resuspended in wash buffer five times before freezing the pellets at −80°C overnight. Pellets were washed twice more to remove endogenous GABA and used at a final concentration of 1 mg/ml. High-affinity ([3H]muscimol (specific activity 30 Ci/mmol) (PerkinElmer Life Sciences, Boston, MA) binding was conducted over a concentration range of 0.5 to 100 nM in a final assay volume of 500 μl and incubated for 90 min at 0–4°C. Nonspecific binding was determined using 100 μM GABA. The reaction was terminated by rapid filtration under vacuum (<25 in. Hg) using GF/B filter strips (Whatman, Maidstone, UK) pretreated with 0.03% polyethyleneimine. Samples were washed twice with 3-ml aliquots of assay buffer at 0–4°C. Filters were dried, added to liquid scintillation cocktail, and counted in a liquid scintillation counter. Saturation binding curves were evaluated using Prism (GraphPad Software, San Diego, CA) to obtain the K<sub>B</sub> and B<sub>max</sub> values and compared between genotypes by one-way ANOVA.

[3H]Zolpidem (specific activity 48 Ci/mmol) binding was determined using 0.125–25 nM; PerkinElmer Life Sciences) binding was determined using 100 μM GABA. The reaction was terminated by rapid filtration under vacuum (<25 in. Hg) using GF/B filter strips (Whatman, Maidstone, UK) pretreated with 0.03% polyethyleneimine. Samples were washed twice with 3-ml aliquots of assay buffer at 0–4°C. Filters were dried, added to liquid scintillation cocktail, and counted in a liquid scintillation counter. Saturation binding curves were evaluated using Prism (GraphPad Software, San Diego, CA) to obtain the K<sub>B</sub> and B<sub>max</sub> values and compared between genotypes by one-way ANOVA.

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buffer (50 mM Tris-HCl, pH 7.4). Samples were centrifuged at 40,000 g for 15 min and resuspended in wash buffer twice before freezing the pellets at −80°C overnight. Pellets were washed twice more before resuspension in assay buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl). The final tissue concentration was 1 mg/ml. Nonspecific binding for [3H]zolpidem and [3H]flunitrazepam studies was determined using 500 nM zolpidem and 1 μM diazepam, respectively (BIOMOL Research Laboratories, Plymouth Meeting, PA). The final assay volume of 500 μl was incubated for 45 min at 0–4°C. The termination of the binding reaction, washing of the membrane filters, counting the radioactivity, and analyzing the data were carried out as described above for [3H]muscimol.

**Autoradiography.** 1-Butylcyclohexylphosphorothionate ([35S]TBPS) and tritium-labeled ethyl-8-azoido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate ([3H]Ro15-4513) were purchased from PerkinElmer Life Sciences. Fumazenil (Ro15-1788) was donated by F. Hoffmann-La Roche (Basel, Switzerland). Picrotoxin was purchased from Sigma-Aldrich (St. Louis, MO). For autoradiography, 14-μm horizontal or frontal serial sections were cut from three to five frozen mouse brains of each genotype using a cryostat (Microm), thaw-mounted onto gelatin-coated glass objects, and stored frozen under desicant at −20°C. All experiments were carried out in parallel manner with respective genotype, eliminating any day-to-day variation in receptor assays between genotype. The autoradiographic procedures for regional localization of [3H]Ro15-4513 and [35S]TBPS binding were as described previously (Makele et al., 1997). 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. The final incubation in the same buffer was performed with 6 nM [35S]TBPS at room temperature for 90 min and assays with 10 nM [3H]Ro15-4513 at 0–4°C for 60 min. After incubation, sections in both assays were washed 3 × 15 s in ice-cold incubation buffer. Sections were then dipped into distilled water, air-dried under a fan at room temperature, and exposed with plastic [3H]methacrylate or [14C]methacrylate standards to Biomax MR films (Eastman Kodak) for 1 to 8 weeks. Nonspecific binding was determined with 10 μM Ro15-1788 and 100 μM picrotoxin in [3H]Ro15-4513 and [35S]TBPS assays, respectively. Images from representative autoradiography films were scanned, processed with Adobe Photoshop (version 3.0; Adobe Systems, Mountain View, CA) and Corel Draw 5.0 programs, and printed for figures. The concentration of [3H]Ro15-4513 (10 nM) was greater than or equal to the dissociation constants for a range of recombinant and native GABA<sub>A</sub> receptors (Pritchett et al., 1989; Ludens et al., 1990; Pritchett and Seeburg, 1990; Wisden et al., 1991). Therefore, the autoradiographic images should represent the density rather than affinity of binding sites. Autoradiography films were quantified using AID image analysis system (Imaging Research, St. Catharines, Ontario, Canada) as described previously (Makele et al., 1997). Binding densities for each brain area were averaged from measurements of one to three sections per 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 [14C]). Significance between the mouse lines in different brain regions was assessed by two-way ANOVA followed by Bonferroni’s post hoc test using Prism.

**Chloride Uptake Assay.** After decapitation, brains were immediately removed and placed in ice-cold saline. Cerebral cortices of seven mice per genotype were pooled for each experiment. Synaptoneurosomes were prepared and Cl<sup>−</sup> uptake was conducted as described previously (Morrow et al., 1988). The synaptoneurosomal pellet was resuspended in 6.6 volumes of ice-cold assay buffer (20 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO<sub>4</sub>, and 2.5 mM CaCl<sub>2</sub>, pH 7.4) for a final protein concentration of approximately 5 mg/ml. The homogenate was aliquoted 200 μl assay tube and preincubated at 30°C for 12 min. Muscimol-stimulated Cl<sup>−</sup> uptake was initiated by addition of 0.2 μCi of [3H]PerkinElmer Life Sciences) in the presence of various concentrations of muscimol (1–20 μM). The solution was vortexed and uptake terminated after 5 s by addition of 4 ml of ice cold assay buffer containing 100 μM picrotoxin with rapid vacuum filtration over G6 filters (Fisher Scientific, Pittsburgh, PA) using a single manifold filter apparatus (Hoeffer, San Francisco, CA). After two more washes, filters were allowed to dry and radioactive counts determined by liquid scintillation spectroscopy. Basal chloride uptake was measured in the absence of muscimol and subtracted from all tubes to determine muscimol-stimulated chloride uptake. Concentration-response curves were evaluated using computerized nonlinear regression (Prism; GraphPad Software) to obtain the EC<sub>50</sub> and E<sub>max</sub> values and compared between genotype by one-way ANOVA.

**Bicuculline-Induced Seizure Threshold Test.** Seizure thresholds were determined at the beginning of the light cycle as described previously (Devaud et al., 1995). Mice were restrained in a Plexiglas plunger-style mouse restraint (Braintrace Scientific, Braintest, MA). Threshold determination was made by constant lateral tail vein infusion of bicuculline (Sigma-Aldrich) dissolved in 0.1 N HCl and diluted with isotonic saline to a final concentration of 0.05 mg/ml, pH 7. The solution was infused at a constant rate of 0.5 ml/min; the endpoint was taken as the first myoclonic jerk of the head and neck. This time point precedes forepaw clonus and generalized tonic/clonic convulsions. Each animal was tested once. Seizure thresholds were determined by experienced observers who were blind to the experimental conditions. Seizure thresholds were calculated from the time of infusion × dose of bicuculline per body weight and presented as milligrams per kilogram of bicuculline. Data were analyzed by one-way ANOVA using Prism.

**Results**

**α1 Subunit Deletion Produces Selective Loss of β2/3** and γ2 Subunit Peptides and Elevations in α2 and α3 Subunit Peptides. Western blot analysis demonstrated the successful elimination of α1 subunit peptide expression in cerebral cortex (Fig. 1) as well as whole brain (Vicini et al., 2001). α1<sup>+/−</sup> and α1<sup>−/−</sup> mice exhibited 39 ± 3% (p < 0.0001) and 100% reductions in α1 subunit peptide expression in the P2 membrane fraction of cerebral cortex, respectively. Because the major GABA<sub>A</sub> receptor subtype containing α1 subunits expressed in brain is α1β2γ2 (Barnard et al., 1998), we measured the effect of the loss of α1 subunit expression on β2/3 and γ2 subunit expression (Fig. 1). Reductions in β2/3 subunit expression (35 ± 4%; p < 0.01; 65 ± 7%; p < 0.001) were observed in α1<sup>+/−</sup> and α1<sup>−/−</sup> mice, respectively. Expression of γ2 subunit peptide was reduced by 47 ± 9% (p < 0.001) in α1<sup>−/−</sup> mice. Adaptations in the expression of other GABA<sub>A</sub> receptor α subunit variants were observed in α1<sup>−/−</sup> mice. There were 37 ± 4% (p < 0.001) and 39 ± 6% (p < 0.001) increases in α2 and α3 subunit peptide expression, respectively, in α1<sup>−/−</sup> mice. No significant differences in α4, α5, or δ subunit peptide expression were observed across genotypes. Data were analyzed from generations F5 to F7 and identical results were obtained (data not shown); therefore, data from all generations were collapsed in Fig. 1.

**Pharmacological and Functional Characterization of α1 Subunit Knockout Mice.** Inhibition of α1 subunit expression resulted in a reduction of GABA<sub>A</sub> receptor density in the membrane fraction of the cerebral cortex. High-affinity [3H]muscimol saturation binding studies revealed a 56 ± 8% reduction (p < 0.005) in binding sites with unaltered affinity (K<sub>D</sub>) in α1<sup>−/−</sup> mice (Fig. 2A). Saturation binding experiments using the direct GABA<sub>A</sub> receptor antagonist [3H]SR-95531 were conducted to verify the apparent loss in receptor number observed with [3H]muscimol binding because agonist binding can vary with activation state of the receptor.
Impaired GABA transmission leads to neuronal hyperexcitability, a condition associated with increased susceptibility to seizure (Macdonald and Olsen, 1994). Bicuculline-induced seizure thresholds were measured to determine seizure susceptibility in α1+/− and α1−/− mice. The latency to which a constant tail vein infusion of the direct GABAA receptor antagonist bicuculline caused seizure activity was measured and used to determine the dose of bicuculline required to meet the threshold to seizure. α1−/− mice exhibited a 37 ± 6% reduction (p < 0.001) in seizure threshold (increased susceptibility to seizure) compared with α1+/− mice (Fig. 6). Although not systematically quantified, α1−/− mice exhibited more severe seizures that usually progressed to tonic clonic convulsions, wild running, clonus, and death. In contrast,


α1+/− mice rarely exhibited tonic clonic convulsions after the determination of seizure threshold.

Discussion

Production of mice with targeted deletion of individual GABA_α receptor subunits has provided valuable insight into the contribution of specific receptor subtypes to CNS mechanisms controlling inhibitory tone, GABA-related disorders, and actions of drugs that act upon GABA_α receptors. Likewise, the initial characterization of the α1+/− mouse line has revealed several interesting phenomena related to α1 expression. Although the mice exhibit reduced GABA_α receptor number and function and adaptations in GABA_α receptor subunit expression, deletion of the α1 subunits resulted in viable offspring with a handling-induced tremor, but maintained normal fertility, litter size, and body weight across several generations. The overtly normal behavior of the mice is remarkable and suggests that the CNS expresses roughly several generations. The overtly normal behavior of the mice suggests that the CNS expresses roughly as many GABA_α receptors as required to maintain normal inhibitory tone. However, handling-induced tremor in α−/− mice may suggest reduced CNS inhibition and increased seizure susceptibility in these mice provides evidence that further blockade of GABA_α receptors severely impairs inhibitory neurotransmission.

The α1−/− mice provide an interesting model to study the assembly of GABA_α receptors. Deletion of the α1 subunit resulted in a dramatic reduction in the number of GABA_α receptors in membrane fractions and brain sections presumably due to loss of the α1 subunit-containing receptors. Loss of the majority of cortical GABA_α receptors as measured by [3H]muscimol, [3H]SR-95531, and [35S]TBPS binding in conjunction with similar magnitude losses of β2/3 and γ2 subunit expression provides further support that α1 subunits are required for the assembly of most α2/3γ2 receptors in brain, despite compensatory increases in α2 and α3 subunit peptide expression. The observed decrease in [3H]flunitrazepam saturation binding and [3H]Ro15-4513 autoradiographic binding is also consistent with this hypothesis. In a previous study, α subunits were also shown to determine receptor assembly in vivo as deletion of α5 subunits in hippocampal pyramidal cells prevented receptor assembly and reduced receptor binding (Fritschy et al., 1997).

Absolute differences in brain regional binding levels were detected by autoradiographic analysis of [35S]TBPS versus [3H]Ro15-4513 binding as described previously (Olsen et al., 1990; Turner et al., 1991; Makela et al., 1997; Korpi et al., 2002). Furthermore, deletion of the α1 subunit differentially altered [35S]TBPS versus [3H]Ro15-4513 within brain regions. These discrepancies are likely due to several factors, including differences in the receptor subtypes labeled by each ligand (Korpi et al., 2002) and differences in the percentage of receptor occupancy of the ligands (based on the concentrations tested). The reduction in [35S]TBPS binding in the α1−/− mice was greater than the reduction in [3H]Ro15-4513 binding in most brain regions. These data may indicate that [3H]Ro15-4513 preferentially labels receptor populations that are less affected by deletion of α1 subunits or those that are compensatorily increased by elevated α2 and α3 subunit expression. [35S]TBPS exhibits weak binding to some of the
α2 and α3 subunit-containing receptors (Lüddens and Korpi, 1995) that would be labeled by [3H]Ro15-4513. Therefore, compensatory increases in α2 or α3 subunit-containing receptors would be expected to diminish the loss of [3H]Ro15-4513 binding more than the loss of [35S]TBPS binding. These data are consistent with the possibility that compensatory increases in α2/α3 subunit-containing receptors are present in α1−/− mice. Clearly, both ligands demonstrate dramatic reductions in GABA<sub>A</sub> receptor binding sites.

Benzodiazepine binding sites, located at the interface of α and γ2 subunits (for review, see Sigel and Buhr, 1997), were severely affected by deletion of the α1 subunit. Complete loss of high-affinity [3H]zolpidem binding in α1−/− mice confirms the selectivity of this ligand for α1 subunit-containing receptors as described in vitro (Pritchett et al., 1989). Previous studies suggest that up to 70% of BDZ binding sites have properties of α1 subunit-containing (type I) receptors (Braestrup and Nielsen, 1981; Niddam et al., 1987; Dennis et al., 1988). However in α1−/− mice, we observed a loss of 46% of [3H]flunitrazepam binding, suggesting that compensation by increased expression of α2 and α3 subunits may have resulted in the increased assembly of α2 and α3 subunit-containing (type II) BDZ receptors. This possibility is consistent with the observation that the decrease in the B<sub>max</sub> of [3H]zolpidem binding (1247 fmol/mg of protein) was greater than the loss of maximal [3H]flunitrazepam binding (827 fmol/mg of protein) in α1−/− mice. Alterations in BDZ pharmacology observed in α1−/− mice would be expected to impact the behavioral actions of BDZs and may aid in defining properties associated with actions at various BDZ receptors.

Elegant studies conducted in knockin mice containing a point mutation at the BDZ binding site of the α subunit have provided models in which the behavioral responses to an array of BDZ site ligands have been selectively attenuated without affecting receptor assembly or sensitivity to GABA (for review, see Rudolph et al., 2001). In α1(H101R) mice, the sedative and amnesic effects of diazepam were ablated, whereas the seizure-protective effect was partially reduced (Rudolph et al., 1999). Similar studies conducted in α2(H101R) and α3(H101R) mice have attributed the anxiolytic effects of diazepam to α2-containing receptors (Low et al., 2000). Together, these studies have begun to dissociate the receptor subtypes responsible for the anxiolytic, sedative, myorelaxant, motor-impairing, hypnotic, amnesic, and ethanol-potentiating effects of BDZs. Furthermore, the pharmacological and behavioral profile of current and future selective BDZs can be examined in these mice (e.g., zolpidem) (Crestani et al., 2000). These results would predict that the α1−/− mice will exhibit a loss of the sedative-hypnotic effects of diazepam and zolpidem with a partial loss in the anticonvulsant effect of diazepam. We have observed a complete loss of the ability of diazepam (1–30 mg/kg) to prevent bicuculline-induced seizures in α1−/− mice (Kralic et al., 2001). Additional studies are underway to further compare these models.

The alterations in muscimol-stimulated 36Cl<sup>−</sup> uptake in

### Table 1: Alterations in Brain Regional Ligand Binding in GABA<sub>A</sub> Receptor Subunit α1<sup>−/−</sup> Mice

<table>
<thead>
<tr>
<th>Ligand/Brain Region</th>
<th>α1&lt;sup&gt;+&lt;/sup&gt;+</th>
<th>α1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>[35S]TBPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>165 ± 17</td>
<td>47.9 ± 7.0</td>
</tr>
<tr>
<td>Thalamus</td>
<td>547 ± 28</td>
<td>82.3 ± 5.0***</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>450 ± 82</td>
<td>201 ± 22***</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>281 ± 59</td>
<td>115 ± 8***</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>895 ± 44</td>
<td>173 ± 7***</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>215 ± 16</td>
<td>107 ± 13*</td>
</tr>
<tr>
<td>[3H]Ro15-4513</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>103 ± 1</td>
<td>77.1 ± 2.6***</td>
</tr>
<tr>
<td>Thalamus</td>
<td>54.1 ± 2.7</td>
<td>19.8 ± 1.5***</td>
</tr>
<tr>
<td>Basal ganglia</td>
<td>49.5 ± 1.1</td>
<td>35.6 ± 1.5***</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>104 ± 1</td>
<td>87.4 ± 1.4***</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>66.2 ± 6.2</td>
<td>24.9 ± 1.6***</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>69.0 ± 1.8</td>
<td>30.4 ± 1.6***</td>
</tr>
</tbody>
</table>

* p < 0.05, Bonferroni post-test.
*** p < 0.001, Bonferroni post-test.
cerebral cortex of α1−/− mice indicate a dramatic reduction in GABAergic tone that may underlie the increased seizure susceptibility in α1−/− mice. Studies in stably transfected mouse L(tk−) cells reported that the maximal effect of muscimol-stimulated chloride flux varies with expression levels of α1, α5, or α6 subunits in conjunction with the β and γ subunits (Harris et al., 1998). Several in vivo studies suggest that the presence of the α1 subunit contributes to the efficacy of GABA agonists. Knockdown of α1 subunits using antisense oligonucleotides resulted in a decrease in GABA-mediated chloride flux (Malatynska et al., 2000). Patch-clamp recordings measured a reduction in the amplitude of evoked inhibitory postsynaptic currents in slices from visual cortex after treatment with α1 antisense oligonucleotides (Brussaard and Baker, 1995). A decrease in α1 subunit mRNA and peptide expression has been associated with a decrease in the EC50 of muscimol-stimulated chloride flux after chronic ethanol administration (Morrow et al., 1988;...
Devaud et al., 1997), whereas there is no change in total receptor number (for review, see Grobin et al., 1998). Although these studies support the hypothesis that α1 subunits contribute to the functional capacity of the receptor, a reduction in GABA\(_A\) receptor number may also contribute to the loss of agonist efficacy. In addition, the reduced potency of muscimol in α1−/− mice suggests that α subunits modulate the potency of muscimol, a property that should not be affected by receptor number, but is consistent with lower GABA sensitivities of α2 and α3 subunit-containing receptors than α1 subunit-containing receptors (Lüddens and Korpi, 1995).

The present study demonstrates that GABA\(_A\) receptor subunit expression and function are altered in the cerebral cortex of α1−/− mice. In a previous study using these mice, Vicini et al. (2001) demonstrated that α1 subunits contribute to the developmental shortening of spontaneous inhibitory post synaptic currents and mIPSCs in stellate cells of the cerebellum. Because GABA\(_A\) receptor binding using \(^{35}S\)TBPS and \(^{3}H\)Ro15-4513 are reduced in cerebellum and throughout brain, it is likely that GABA\(_A\) receptor function, subunit expression, and assembly are also altered in many brain regions. Studies are underway to further investigate this possibility.

The decrease in bicuculline-induced seizure threshold measured in α1−/− mice suggests that α1 subunit-containing receptors influence seizure susceptibility through maintenance of inhibitory tone. As described earlier, a strong association between the expression of the α1 subunit and seizure susceptibility has been drawn from developmental, epileptic, and alcohol dependence and withdrawal models. The developing brain expresses predominately GABA\(_A\) receptor α2, α3, and α5 subunits during embryonic and early postnatal stages shifting to high levels of α1 subunit expression in adult brain (Poulter et al., 1992; Laurie et al., 1992). This switch in the expression pattern of α subunits during brain development is associated with a decreased seizure susceptibility in adult brain (Aicardi and Chevrie, 1970; Mecarelli et al., 1988). Moreover, models of seizure-prone and -resistant mice express 50% less and 200% more α1 subunit than controls, respectively (Poulter et al., 1999). Withdrawn ethanol-dependent rats exhibit increased seizure susceptibility and a concomitant reduction in α1 subunit expression (Devaud et al., 1997). These findings support the hypothesis that α1 subunit expression serves a critical role in regulating CNS excitability and seizure susceptibility and a potential target for anticonvulsant drug therapy.

Recently, production of an α1 subunit knockout mouse (MSD mice; Sur et al., 2001) was achieved using a different gene targeting method (Sur et al., 2001; Vicini et al., 2001). Although both mouse lines share mostly similar phenotypes, they also differ in several ways. In addition to the successful deletion of the α1 subunit and production of viable knockout mouse lines, both lines exhibited similar changes in GABA\(_A\) receptor pharmacology and an absence of any overt behavioral differences. Furthermore, both lines displayed increased peptide expression of α2 and α3 subunits. Although these adaptations were consistent across generations in our mouse line, the adaptations were gradually lost in successive generations of MSD mice (Sur et al., 2001). This difference is likely due to distinct gene-targeting methods or breeding strategies used. Interbreeding of α1−/− mice may have resulted in the smaller litter sizes observed in MSD mice in earlier generations. Litter sizes may have recovered in size due to selection of knockout mice able to survive α1 gene deletion or those containing adaptations in α2 and α3 subunit expression. Measurement of mRNA revealed no adaptations in β3 and γ2 expression in MSD mice (Sur et al., 2001), whereas β2/3 and γ2 peptide expression was significantly reduced in our mice, suggesting either another inherent difference between lines or adaptations in subunit expression mediated by translational control or assembly mechanisms. Although functional studies conducted in both mouse lines used different methods and targeted different brain regions, they collectively showed that GABA\(_A\) receptor function is diminished in the absence of α1 subunit expression. Differences between these mouse lines may be inherent to the line or due to varying techniques used for breeding and investigation. Together, these independent studies provide a thorough examination of the role of the α1 subunit in GABA\(_A\) receptor pharmacology, function, and related behaviors.

In conclusion, we have shown that global deletion of the α1 subunit of the GABA\(_A\) receptor results in reduced agonist-induced receptor function, BZD pharmacology, and increased seizure susceptibility without affecting viability or overt behavior. The increased seizure susceptibility of α1−/− mice supports the role of α1 subunit expression in the maintenance of CNS inhibitory tone suggested by models of epilepsy and alcohol withdrawal. Adaptations in GABA\(_A\) receptor subunit expression provide further evidence for plasticity in the GABAergic system. Results from the present studies suggest a specific role for α1 subunit-containing receptors in GABAergic neurotransmission and extend previous findings supporting the α1 subunit-containing receptor as a target for the development of GABA\(_A\) receptor subtype-specific drugs, e.g., for new antiepileptic/anticonvulsant drugs. Our new mouse model will be useful in the study of mechanisms that regulate GABA\(_A\) receptor assembly as well as testing possible new drug entities. Furthermore, important pharmacodynamic studies remain such as determining the response of α1−/− mice to the actions of benzodiazepines, alcohol, volatile anesthetics, barbiturates, and neurosteroids, all of which act upon GABA\(_A\) receptors.

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References


