

TOLERANCE TO DIAZEPAM AND CHANGES IN GABA_A RECEPTOR SUBUNIT EXPRESSION IN RAT NEOCORTICAL AREAS

C. PESOLD,* H. J. CARUNCHO,† F. IMPAGNATIELLO,* M. J. BERG,‡
J.-M. FRITSCHY,§ A. GUIDOTTI* and E. COSTA*||

*University of Illinois at Chicago, The Psychiatric Institute, 1601 West Taylor Street, M/C 912,
Chicago, IL 60612, U.S.A.

†Department of Morphological Sciences, University of Santiago de Compostela School of Medicine,
Rua San Francisco s/n, 15705, Santiago de Compostela, Spain

‡Center for Neurochemistry, The Nathan Kline Institute for Psychiatric Research, Bldg 39,
140 Old Orangeburg Road, Orangeburg, NY 10962, U.S.A.

§Institute of Pharmacology, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich,
Switzerland

Abstract—Long-term treatment with diazepam, a full allosteric modulator of the GABA_A receptor, results in tolerance to its anticonvulsant effects, whereas an equipotent treatment with the partial allosteric modulator imidazenil does not produce tolerance. Use of subunit-specific antibodies linked to gold particles allowed an immunocytochemical estimation of the expression density of the α_1 , α_2 , α_3 , α_5 , $\gamma_{2L\&S}$ and $\beta_{2/3}$ subunits of the GABA_A receptor in the frontoparietal motor and frontoparietal somatosensory cortices of rats that received long-term treatment with vehicle, diazepam (three times daily for 14 days, doses increasing from 17.6 to 70.4 $\mu\text{mol/kg}$), or imidazenil (three times daily for 14 days, doses increasing from 2.5 to 10.0 $\mu\text{mol/kg}$). In this study, tolerance to diazepam was associated with a selective decrease (37%) in the expression of the α_1 subunit in layers III–IV of the frontoparietal motor cortex, and a concomitant increase in the expression of the α_5 (150%), $\gamma_{2L\&S}$ and $\beta_{2/3}$ subunits (48%); an increase in α_5 subunits was measured in all cortical layers. In the frontoparietal somatosensory cortex, diazepam-tolerant rats had a 221% increase in the expression of α_5 subunits in all cortical layers, as well as a 35% increase in the expression of α_3 subunits restricted to layers V–VI. Western blot analysis substantiated that these diazepam-induced changes reflected the expression of full subunit molecules. Rats that received equipotent treatment with imidazenil did not become tolerant to its anticonvulsant properties, and did not show significant changes in the expression of any of the GABA_A receptor subunits studied, with the exception of a small decrease in α_2 subunits in cortical layers V–VI of the frontoparietal somatosensory cortex.

The results of this study suggest that tolerance to benzodiazepines may be associated with select changes in subunit abundance, leading to the expression of different GABA_A receptor subtypes in specific brain areas. These changes might be mediated by a unique homeostatic mechanism regulating the expression of GABA_A receptor subtypes that maintain specific functional features of GABAergic function in cortical cell layers. © 1997 IBRO. Published by Elsevier Science Ltd.

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Molecular cloning has so far identified 14 genes encoding GABA_A receptor subunits, grouped in four families: α_{1-6} , β_{1-4} , γ_{1-3} and δ .^{51,64} Pentameric assemblies of these subunits form GABA_A receptor subtypes with characteristic pharmacological and physiological properties.^{2,15,49,56} Classical benzodiazepines such as diazepam express anticonvulsant, anxiolytic, hypnotic, myorelaxant and sedative

properties via high-affinity binding to a recognition site located on GABA_A receptors.⁶⁰ Although the benzodiazepine binding site is located on the α subunit, the molecular nature of the γ subunits expressed in the pentameric assembly influences this binding and is involved in determining the direction of the modulatory action of benzodiazepines at GABA-operated Cl⁻ channels.^{25,38,64} In contrast, the molecular structure of the β subunit included in any given GABA_A receptor subtype does not appear to play a major role in defining the extent of benzodiazepine-induced amplification of GABA_A-elicited Cl⁻ current intensity.^{12,15,26,38,55} When different benzodiazepines were studied for their ability to amplify the intensity of GABA-elicited Cl⁻

||To whom correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; FAM, full allosteric modulator; FrPaM, frontoparietal motor; FrPaSS, frontoparietal somatosensory; PAM, partial allosteric modulator; PBS, phosphate-buffered saline; SAM, selective allosteric modulator; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline–Tween.

currents at structurally different recombinantly expressed GABA_A receptors,^{15,34,38,56} it was found that the benzodiazepine intrinsic modulatory action is highly dependent on the molecular nature of the α and the γ subunits that participate in the GABA_A receptor assembly. Three classes of anxiolytics acting on benzodiazepine binding sites of the GABA_A receptors have been characterized:¹³ (i) full allosteric modulators (FAM), which maximize GABA action in a large number of GABA_A receptors; (ii) selective allosteric modulators (SAM), which maximize GABA action in a selected number of GABA_A receptors; and (iii) partial allosteric modulators (PAM), which never maximize GABA action and usually amplify the intensity of GABA-elicited Cl⁻ currents in most recombinant and native receptors, but only by a modest extent. According to these studies, most benzodiazepines amplify the intensity of GABA currents better in receptors that include the α_1 or α_3 subunits, than in those including α_5 subunits.

Long-term repeated daily administrations of benzodiazepines with FAM profiles (e.g., diazepam, triazolam) rapidly induce tolerance to some of their pharmacological properties, whereas administration of those with PAM profiles (e.g., imidazenil), even in doses several-fold that of equipotent doses of diazepam that elicit tolerance, do not cause tolerance liability.^{1,24,45} While there is a considerable amount of biochemical and electrophysiological evidence indicating that tolerance to the anticonvulsant effect of diazepam is associated with a down-regulation of GABA_A receptor function,^{21,22,28,29,33,57} the precise molecular mechanism underlying this GABAergic down-regulation is not presently understood.

Considerable evidence is emerging that benzodiazepine tolerance associated with chronic exposure to diazepam, flurazepam or lorazepam is a consequence of regulatory changes in the expression of α and γ GABA_A receptor subunits which modify the abundance of GABA_A receptor subtypes expressed in different specific brain areas.^{27,32,50,54,61,65} In a recent study, we have reported that rats treated three times daily for 14 days with increasing doses of diazepam became tolerant to its anticonvulsant action.¹ Quantitative measurements of mRNA encoding for GABA_A receptor subunits using a competitive polymerase chain reaction assay revealed that diazepam-tolerant rats had a significant reduction in the expression of mRNA encoding for the α_1 and the γ_2 subunits, and a significant increase in the expression of mRNA encoding for the α_5 subunit in the frontoparietal motor (FrPaM) cortex, but not in the frontoparietal somatosensory (FrPaSS) cortex.³⁰ In contrast, rats treated with equipotent doses of the PAM imidazenil exhibit neither tolerance nor changes in the expression of any of the mRNAs encoding for GABA_A receptor subunits in the same neocortical regions. The results of these studies suggest that tolerance to benzodiazepines may be linked with an alteration in the neocortical expression of

various GABA_A receptor subunits.³⁰ The interpretation of these results is somewhat limited by the fact that most studies failed to measure subunit expression and only measured changes in the expression of mRNA encoding various GABA_A receptor subunits, which may not necessarily reflect modifications in the steady-state expression of their respective translation products.^{7,63} The few studies that have examined the effects of other pharmacological challenges on GABA_A receptor subunit expression revealed that changes in mRNA are not always paralleled by changes in their respective translation product. For instance, the changes in GABA_A receptor subunit mRNA expression observed following a protracted exposure to GABA of mouse primary cultures of cortical neurons were paralleled by changes in the subunit protein expression.⁴⁴ In contrast, the changes in subunit protein expression in rats receiving a protracted ethanol treatment⁴² were not necessarily accompanied by any changes in the expression of their respective mRNA.^{43,48} Finally, in rat cerebellar granule cells exposed to flumazenil, the changes in the expression of subunit proteins were sometimes either dissociated from a change in their respective mRNA expression or associated with mRNA changes in the opposite direction.⁶⁶ The frequent discordance between activity-dependent changes in GABA_A receptor subunit mRNA and protein expression is not surprising because of the temporal delay between the onset of changes in mRNA expression and in their translation products. It is also possible that not all proteins detected by immunolabelling techniques are functionally operative, and that protein expression depends on translation rate and mRNA stability, rather than DNA transcription rates, exclusively. Moreover, there are also topographical differences between mRNA and protein expression, with proteins expressed on soma, axons and dendrites of various neurons, whereas mRNA expression is almost exclusively restricted to cell bodies.

There are only preliminary reports of studies that have examined the expression of GABA_A receptor subunit proteins during benzodiazepine tolerance.³⁰ The evidence that expression of mRNAs encoding for GABA_A receptor subunits changes during benzodiazepine tolerance, coupled with the frequent lack of correlation between activity-dependent changes in GABA_A receptor subunit mRNAs and that of their respective protein expression, underlines the necessity of establishing that changes in the expression of GABA_A receptor subunits are also associated with tolerance to benzodiazepines.

Using antibodies directed specifically against α_1 , α_2 , α_3 , α_5 , γ_2 and $\beta_{2/3}$ subunits of the GABA_A receptor, the present immunocytochemical study has measured the expression density of several GABA_A receptor subunits in the FrPaM and the FrPaSS cortices of rats made tolerant to the anticonvulsant effects of diazepam on bicuculline-induced seizures. The expression of GABA_A receptor subunits

following protracted treatment with this FAM was compared with that following an equipotent treatment of identical duration with the PAM imidazenil, which does not elicit tolerance, in order to eliminate pharmacological action *per se*, rather than tolerance as the cause of any change in subunit expression.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley rats weighing 125–135 g (Zivic Miller, Pittsburg, PA, U.S.A.) were housed three per cage and maintained on a 12-h light/dark cycle, with food and water available *ad libitum*. All experiments were carried out in accordance with the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 80-23), revised 1978. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drug-treatment schedule

Diazepam or imidazenil (Hoffman-La Roche, Nutley, NJ, U.S.A.) were suspended in vehicle (distilled water containing 0.05% Tween 20) and administered by oral gavage in a 2-ml volume, three times per day (at approximately 09.00, 14.00 and 19.00) for 14 consecutive days with the following dose regimens: diazepam (day 1–3, 17.6 $\mu\text{mol/kg}$; day 4–6, 35.2 $\mu\text{mol/kg}$; day 7–10, 52.8 $\mu\text{mol/kg}$; day 11–14, 70.4 $\mu\text{mol/kg}$) and imidazenil (day 1–3, 2.5 $\mu\text{mol/kg}$; day 4–6, 5.0 $\mu\text{mol/kg}$; day 7–10, 7.5 $\mu\text{mol/kg}$; day 11–14, 10.0 $\mu\text{mol/kg}$). Equivalent anticonvulsant doses of diazepam and imidazenil, and optimal rates of dose escalation to produce anticonvulsant tolerance were determined in previous experiments in our laboratory.^{1,46} Control rats received only vehicle for the same 14-day administration schedule.

In a parallel study,³⁰ 24 h after termination of this drug regimen, diazepam showed anticonvulsant effect against bicuculline-induced seizures in vehicle-treated animals by increasing the threshold dose of bicuculline necessary to elicit convulsions from 1.3 $\mu\text{mol/kg}$ to 3.2 $\mu\text{mol/kg}$. In long-term diazepam-treated rats, however, diazepam no longer protected against bicuculline-induced seizures, with a threshold convulsant dose of bicuculline (1.2 $\mu\text{mol/kg}$) no different from that of rats that did not receive diazepam (i.e. tolerance). Conversely, tolerance did not develop to the anticonvulsant effects of imidazenil following long-term treatment since the ability of imidazenil to raise the threshold dose of bicuculline necessary to elicit convulsions was no different in rats treated chronically with imidazenil (3.4 $\mu\text{mol/kg}$) than in vehicle-treated rats (3.3 $\mu\text{mol/kg}$). Furthermore, there was no cross-tolerance between diazepam and imidazenil, as indicated by diazepam's continued ability to protect imidazenil-treated rats against bicuculline-induced seizures, and similarly imidazenil's continued anticonvulsant action in diazepam-treated rats.^{1,30}

Antibodies

Affinity-purified rabbit polyclonal antisera were used to recognize an amino acid sequence corresponding to residues 1–16 of the α_1 subunit of the GABA_A receptor.¹⁸ Guinea-pig polyclonal antisera raised against subunit-specific peptides were used to recognize residues 1–9 of the α_5 ,^{20,39} residues 1–15 of the α_3 ,⁴ residues 1–10 of the α_5 ¹⁹ and residues 1–29 of the γ_2 subunits of the GABA_A receptor.¹⁹ Finally, mouse monoclonal antibody bd-17 directed against the β -chain of the GABA_A receptor recognizes a common epitope of the β_2 and β_3 subunits,^{16,17} and was obtained commercially from Boehringer Mannheim (Indianapolis, IN, U.S.A.). The specificity of these antibodies was demon-

strated by western blot analysis (see Fig. 4), and immunohistochemically in label-fracture experiments on HEK-293 cells transfected with cDNAs encoding for specific GABA_A receptor subunits.^{9,10,11,15} Goat anti-rabbit IgG, goat anti-guinea-pig IgG and goat anti-mouse IgG secondary antibodies conjugated to 1 nm colloidal gold particles were purchased from Goldmark Biologicals (Phillipsburg, NJ, U.S.A.).

Western blot analysis

Twenty-four hours following the last day of their respective drug treatment regimen, rats were decapitated and their FrPaM cortex was immediately dissected out and frozen. The tissue was homogenized in 20 volumes of cold 0.32 M sucrose (in 10 mM Tris, pH 7.6) with 10 manual strokes using a Teflon homogenizer followed by two 10-s Polytron bursts, 15 s apart. Following centrifugation at 1000 g for 10 min, the supernatant was centrifuged at 100,000 g for 1 h to obtain a crude synaptic membrane fraction. Varying concentrations of protein for a vehicle- or a diazepam-treated rat (12 μg , 8 μg , and 4 μg) were applied to a 10–20% Lemli sodium dodecyl sulfate (SDS) gradient polyacrylamide gel. One-dimensional SDS electrophoresis was performed, followed by transfer to polyvinylidene fluoride membranes. Blots were rinsed with Tris-buffered saline containing 0.1% Tween 20 (TBST) and blocked with 0.2% casein in TBST for 1 h at 37°C while shaking, before a 2-h incubation at room temperature while shaking in primary antibody diluted in 0.2% casein in TBST for several GABA_A receptor subunits α_5 , 1:10,000, γ_2 , 1:5000, $\beta_{2/3}$, 10 $\mu\text{g/ml}$, as well as for the housekeeping protein β -tubulin (1 $\mu\text{g/ml}$; Boehringer Mannheim). Following several rinses in 0.2% casein in TBST, blots were incubated with the appropriate secondary antibody conjugated to alkaline phosphatase diluted in 0.2% casein in TBST (anti-guinea-pig 1:3000 from Sigma; anti-rabbit 1:7500; and anti-mouse 1:5000 from Promega) for 1 h at room temperature while shaking. Following several rinses with 0.2% casein in TBST, blots were rinsed and incubated several times for 5 min while shaking in 0.1 M diethanolamine buffer with 2.5 mM MgCl (pH 10), after which they were incubated in the diethanolamine containing 2.5 mM CSPD chemiluminescent substrate (Tropix, Bedford, MA, U.S.A.) for 30 min before being exposed to film. The membrane was stripped for 1 h (0.2 M glycine-HCl, pH 2.2; 0.1% SDS; 1% Tween 20), then reprobed for each antibody using the same protocol.

Immunocytochemical procedures

Following 14 days of treatment with their respective drug regimens, rats were left drug free for 24 h before being anesthetized with 3 ml/kg of Equithesin (43 ml H₂O+4.25 g chloral hydrate+1.125 g MgSO₄+26.6 ml propylene glycol+12.5 ml ethanol+18.0 ml Nembutal) and perfused intracardially with 100 ml of phosphate-buffered saline (PBS), followed by 100 ml fixative (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M PBS at pH 7.2). Brains were removed and kept overnight in fixative, then embedded in PBS containing 30% sucrose for three days at 4°C. Brains were frozen and 70- μm coronal sections from the FrPaM and the FrPaSS cortices were cut with a cryostat, maintained in PBS for two days at 4°C to remove the glucose embedding, and incubated at room temperature in RPMI 1640 (GIBCO) for 30 min, followed by 30 min in 1% bovine serum albumin (BSA) in PBS. The sections were then incubated overnight at 4°C followed by 2 h at room temperature with the primary antibody diluted in 1% BSA (α_1 , 20,000; α_2 , 1:10,000; α_3 , 1:10,000, α_5 , 1:3000, γ_2 , 1:5000; $\beta_{2/3}$, 1 $\mu\text{g/ml}$), rinsed twice for 30 min in 1% BSA, then incubated for 60 min at room temperature with the corresponding gold-labelled secondary antibody [goat anti-rabbit (α_1), goat anti-guinea-pig (α_2 , α_3 , α_5 , γ_2), goat anti-mouse ($\beta_{2/3}$)], diluted 1:200 in 1% BSA (pilot studies were conducted to

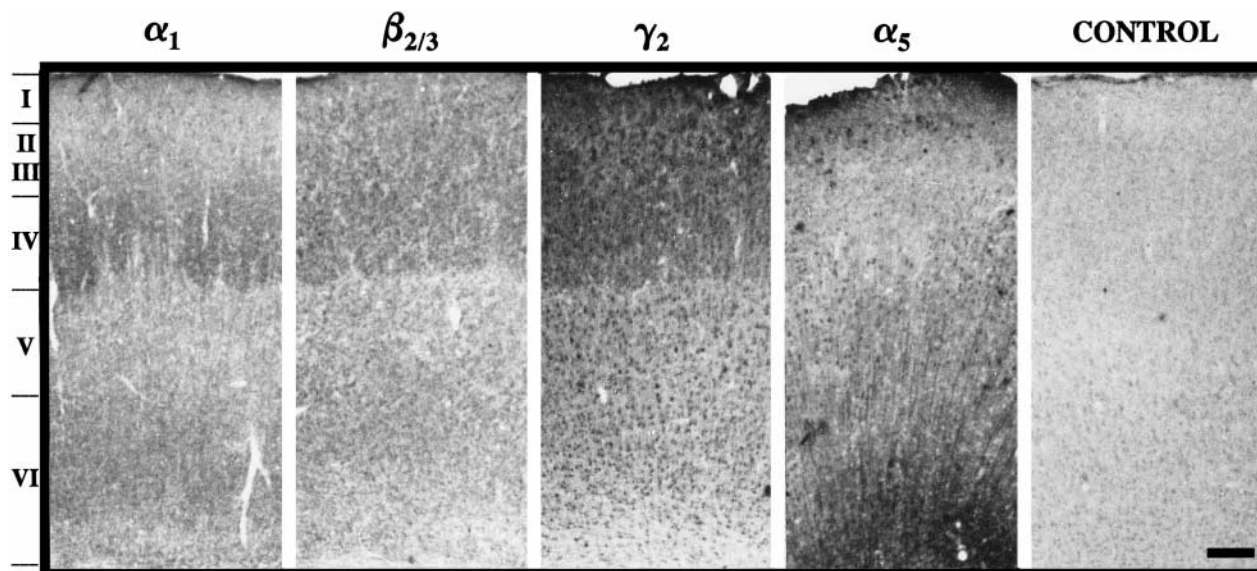


Fig. 1. Photomicrograph showing the laminar distribution of four GABA_A receptor subunits in sections through the frontoparietal somatosensory cortex at the anterior-posterior level of bregma. The colocalization of the α_1 , $\beta_{2/3}$ and γ_2 GABA_A receptor subunits is evidenced by their similar distribution pattern: these three subunits show diffuse labelling of the neuropil which is most intense in cortical layers I, III–IV and VI, with very light labelling in cortical layer V. The α_5 subunit shows a very different pattern of distribution, with lowest labelling of layers II–IV and intense labelling of layers V and VI. The control section depicts the low level of non-specific labelling obtained in sections that received a secondary antibody (in this case goat anti-mouse) but no primary antibody. In all sections, cell bodies were stained with Toluidine Blue. Scale bar=200 μ m.

determine the optimal concentrations of primary and secondary antibodies). Sections were then rinsed four times for 30 min in 1% BSA and once in distilled water, and then treated in the dark with a silver enhancing solution for 20 min (Goldmark Biologicals). The sections were rinsed several times in distilled water overnight, collected on to microscope slides, air dried, counterstained with Toluidine Blue, and photographed using an Olympus BHS microscope equipped with a PM10-ADSP photomicrographic system. For the control sections from which non-specific labelling was determined, an identical protocol was followed except that 1% BSA in PBS was substituted for the primary antibody. Photomicrographs illustrating the immunogold labelling of α_1 , $\beta_{2/3}$, γ_2 and α_5 GABA_A receptor subunits in the FrPaSS cortex are shown in Fig. 1. Note the distinct laminar distribution of these individual subunits, as evidenced by the heterogeneous immunogold labelling density among the various cortical layers.

Analysis of subunit protein expression

The density of colloidal gold particles was calculated stereologically by counting the average number of gold particles in square grids representing 100 μ m² superimposed on to photomicrographs (total labelling). Non-specific labelling was determined by counting the number of gold particles per 100 μ m² in control (no primary antibody) sections. Background labelling was corrected by subtracting non-specific labelling from the corresponding total labelling. Average gold counts were obtained from layers I–II, III–IV and V–VI of the FrPaM and the FrPaSS regions of the cortex, 2.2 mm anterior to bregma, in at least four rats from each group for each antibody used. Specific labelling in each area was compared across the different drug-treatment groups using analysis of variance followed by Fisher's Least Significant Difference pairwise comparisons ($\alpha=0.01$).

Saturation curves for the α_1 and the α_5 subunits, determined in 42- μ m sections of the FrPaM cortex of normal rats

using the same protocol with increasing concentrations of the α_1 (no antibody, 1:80,000, 1:40,000, 1:20,000, 1:10,000, 1:5000) and the α_5 antibodies (no antibody, 1:10,000, 1:6000, 1:3000, 1:1000, 1:500), showed that the α_1 subunit is more abundant than the α_5 subunit. While the use of saturating concentrations of antibody would enable comparisons of the densities of different subunits in any given brain area, the dense expression of some of the GABA_A receptor subunits in the FrPaM and the FrPaSS cortices hampers accurate counting of the number of gold particles. For accurate calculations of the density of labelled subunits in these rat brain regions, subsaturating concentrations of the antibodies were used. While this approach does not lend itself well to comparisons between subunits, it is ideal for investigating possible changes in subunit expression in any given brain area following different drug treatments. Moreover, the reproducibility of the results obtained using this method is quite impressive; when the same protocol was repeated in different groups of animals across different experiments, the interexperimental variability in the density of specific gold particles (i.e. total labelling minus background labelling) was minimal (Table 1).

RESULTS

Immunolabelling of GABA_A receptor subunits in the rat frontoparietal motor and frontoparietal somatosensory cortices

As shown in Fig. 2, rats made tolerant to diazepam showed significant changes in most of the GABA_A receptor subunits studied in the FrPaM cortex, as well as in the expression of selected GABA_A receptor subunits in the FrPaSS cortex, whereas rats that received an equipotent regimen of imidazenil showed virtually no significant change in GABA_A receptor

Table 1. Interexperimental variability of gold immunolabelling by antibodies specific for several GABA_A receptor subunits

GABA _A receptor subunit	α_1	α_3	α_5	γ_2
Experiment 1	29 ± 1.8	—	11 ± 2.0	—
Experiment 2	26 ± 5.1	26 ± 3.9	9.5 ± 1.3	13 ± 1.6
Experiment 3	27 ± 1.3	26 ± 1.0	12 ± 1.8	13 ± 1.1

Mean (± S.E.M.) number of specific immunogold labelling (total minus non-specific) for the α_1 , α_3 , α_5 and γ_2 GABA_A receptor subunits in 100 μm^2 of 70- μm sections taken from the frontoparietal motor cortex of different groups of rats in three separate experiments. The insignificant variability across experiments demonstrates the impressive reproducibility of this gold immunolabelling technique.

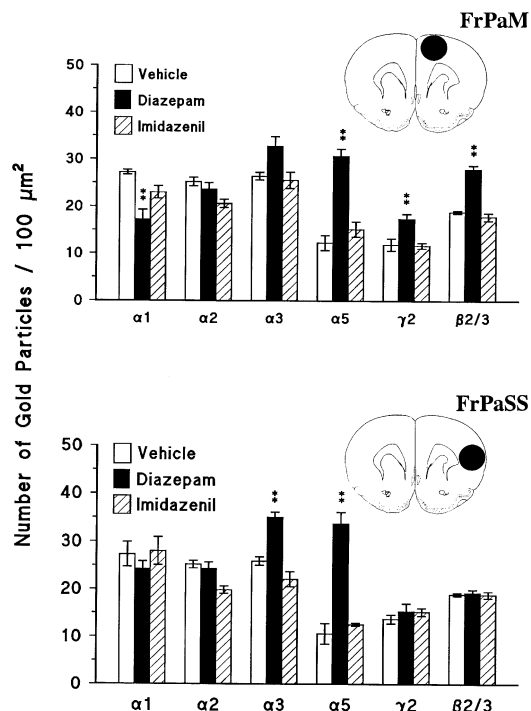


Fig. 2. Mean (± S.E.M.) number of gold particles labelling the α_1 , α_2 , α_3 , α_5 , γ_2 and $\beta_{2/3}$ subunits of the GABA_A receptor in 100 μm^2 of 70- μm sections taken from the frontoparietal motor (top) and the frontoparietal somatosensory cortices (bottom) of rats treated three times daily for 14 days with either vehicle (open bars), diazepam (solid bars) or imidazenil (hatched bars). Shaded circles represent the region where gold immunolabelling was measured and averaged across all cortical laminae.

subunit expression in both areas. The photomicrographs in Fig. 3 depict examples of the changes in immunolabelling density for the α_1 and α_5 subunits in layer IV of the FrPaM cortex of diazepam-tolerant rats.

Significant changes in the expression of the α_1 , γ_2 and $\beta_{2/3}$ subunits were only found in the FrPaM cortex. In this cortical region, tolerance to diazepam

was associated with a 37% decrease in the α_1 subunit, and a 48% increase in both the γ_2 and the $\beta_{2/3}$ subunits, as well as a 150% increase in the α_5 subunit (Fig. 2). The changes in the α_1 and the γ_2 subunits were only statistically significant in layers III–IV, whereas the increases in the $\beta_{2/3}$ and the α_5 subunits were pronounced in all layers of the FrPaM cortex (Table 2). In the FrPaSS cortex, fewer changes in GABA_A receptor subunits were found. In this cortical region, there was a 221% increase in the α_5 subunit, which was pronounced in all cortical layers, as well as a 35% increase in the α_3 subunit expression, which was only significant in cortical layers V–VI (Fig. 2, Table 3). No changes were observed in the expression of the α_2 subunit in either layer of the FrPaM or the FrPaSS cortex during tolerance to the anticonvulsant effects of diazepam.

Long-term treatment with equipotent doses of imidazenil was not associated with significant changes in the expression of any of the GABA_A receptor subunits studied, with the exception of a small (22%) but significant decrease in the expression of the α_2 subunit in cortical layers V–VI of the FrPaSS cortex (Table 3).

The selectivity of these cortical changes in GABA_A receptor subunits following diazepam tolerance is further exemplified by the fact that they were restricted to the area of the FrPaM and FrPaSS cortex located approximately 2 mm anterior to bregma. No such changes were observed in the more posterior regions of the FrPaM or FrPaSS cortex near bregma (data not shown). It is important to note that since the immunogold labelling densities reported in Tables 2 and 3 were obtained from the frontoparietal cortex 2 mm anterior to bregma, where the laminar distribution of the subunits is less distinct than in the more posterior cortical regions, and since they are averaged over two adjacent layers with dissimilar distribution densities, the gold particle density reported in Tables 2 and 3 does not strictly follow the laminar distribution shown in Fig. 1.

Western blot analysis

To ensure that the increases in the immunoreactivity observed immunocytochemically for the α_5 , γ_2 and $\beta_{2/3}$ subunits in the FrPaM cortex were not simply due to increased labelling of non-specific immunoreactive proteins or of metabolites of the target protein, western blot analysis was performed on crude synaptic membranes obtained from this cortical area of rats chronically treated with either vehicle or diazepam. As shown in Fig. 4, the antibody for α_5 immunolabelled a protein band of roughly 55,000 mol. wt, which corresponds to the previously reported size of the α_5 subunit.^{40,47} Significantly, no other bands were immunoreactive for the α_5 subunit antibody, demonstrating both the specificity of this antibody and the specificity of the increase in immunoreactivity observed immunocytochemically.

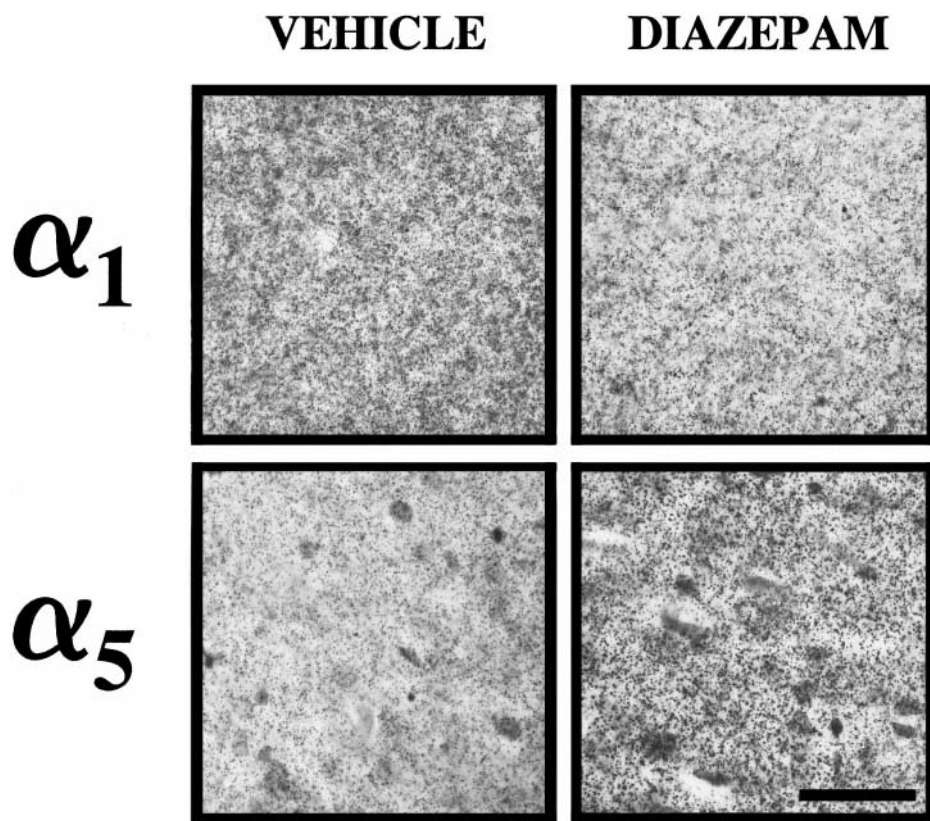


Fig. 3. Photomicrograph showing the gold immunolabelling of the α_1 and α_5 subunits of the GABA_A receptor in 70- μ m sections taken from the frontoparietal motor cortex of rats that received long-term treatment with either vehicle or diazepam. Note the decrease in α_1 subunit labelling and the increase in α_5 subunit labelling in diazepam-tolerant rats. Cell bodies are stained with Toluidine Blue. Scale bar=40 μ m.

The antibody for γ_2 also demonstrated specificity by immunolabelling of only one band of 43,000 mol. wt, which corresponds to the previously reported size of the γ_2 subunit.⁶ Blotting the same membrane with the $\beta_{2/3}$ antibody revealed immunoreactivity at approximately 57,000 mol. wt, which presumably corresponds to the previously reported size of 56 and 58,000 mol. wt for both the β_2 and β_3 subunits.⁶ With this bd-17 antibody, an additional band of lower molecular mass was also labelled (not shown here). Whether this low molecular weight immunoreactive protein band represents a $\beta_{2/3}$ subunit metabolite or an unrelated cross-reacting protein species is unclear at this time. Importantly, it was equally labelled in the vehicle- and the diazepam-treated rats, suggesting that this metabolic product is probably not the source of the increased immunoreactivity observed immunocytochemically.

As shown in Fig. 4, the β -tubulin antibody elicited a single immunoreactive band of 55,000 mol. wt at each concentration of protein for both the vehicle- and the diazepam-treated rats. Despite the equivalent loading of brain homogenates from vehicle and diazepam-treated rats, as documented by the similar labelling intensity of this housekeeping protein, the labelling of the α_5 , γ_2 and $\beta_{2/3}$ subunits visually

Table 2. Immunolabelling density of GABA_A receptor subunits expressed in the frontoparietal motor cortex of vehicle, diazepam or imidazenil-treated rats

Subunit	Cortical layers	Vehicle	Diazepam	Imidazenil
α_1	I-II	27 ± 2.3	19 ± 2.2	22 ± 1.8
	III-IV	31 ± 1.0	17 ± 2.7**	22 ± 0.9
	V-VI	23 ± 3.0	17 ± 2.8	25 ± 2.8
α_2	I-II	25 ± 1.8	24 ± 1.5	21 ± 1.3
	III-IV	27 ± 1.2	26 ± 0.8	23 ± 0.6
	V-VI	24 ± 1.1	21 ± 2.0	18 ± 1.8
α_3	I-II	32 ± 1.6	39 ± 2.4	29 ± 2.8
	III-IV	24 ± 1.9	30 ± 1.6	27 ± 1.0
	V-VI	23 ± 0.4	30 ± 2.9	21 ± 2.3
α_5	I-II	13 ± 0.7	35 ± 2.9**	18 ± 2.7
	III-IV	13 ± 3.0	28 ± 1.7**	14 ± 1.1
	V-VI	12 ± 1.6	29 ± 1.3**	14 ± 1.8
γ_2	I-II	15 ± 1.7	20 ± 0.8	14 ± 1.6
	III-IV	12 ± 1.6	19 ± 1.0**	12 ± 0.7
	V-VI	8.8 ± 1.6	14 ± 1.8	9.4 ± 1.4
$\beta_{2/3}$	I-II	20 ± 0.4	30 ± 0.5**	18 ± 0.9
	III-IV	20 ± 0.6	28 ± 1.7**	18 ± 1.9
	V-VI	17 ± 0.8	26 ± 1.0**	17 ± 1.1

Mean (\pm S.E.M.) number of immunogold particles per 100 μ m² of 70- μ m sections taken from the frontoparietal motor cortex of rats exposed to long-term treatment with either vehicle, diazepam or imidazenil. Statistical significance was determined by analysis of variance followed by Fisher's Least Significant Difference test (** p <0.01).

Table 3. Immunolabelling density of GABA_A receptor subunits expressed in the frontoparietal somatosensory cortex of vehicle, diazepam or imidazenil-treated rats

Subunit	Cortical layers	Vehicle	Diazepam	Imidazenil
α_1	I-II	27 ± 2.9	24 ± 3.3	30 ± 3.5
	III-IV	26 ± 3.3	23 ± 2.1	30 ± 2.4
	V-VI	30 ± 5.2	25 ± 2.1	26 ± 3.8
α_2	I-II	29 ± 0.8	27 ± 2.3	21 ± 1.4
	III-IV	26 ± 0.6	26 ± 1.0	19 ± 1.4**
	V-VI	21 ± 2.2	20 ± 1.6	20 ± 0.4
α_3	I-II	32 ± 2.3	41 ± 1.6	30 ± 2.2
	III-IV	27 ± 2.5	37 ± 2.2	21 ± 2.7
	V-VI	19 ± 1.2	27 ± 1.9**	16 ± 1.3
α_5	I-II	13 ± 2.2	44 ± 3.9**	14 ± 2.0
	III-IV	9.1 ± 2.4	30 ± 3.4**	11 ± 0.9
	V-VI	9.2 ± 2.1	28 ± 3.0**	13 ± 0.7
γ_2	I-II	18 ± 0.7	18 ± 2.0	18 ± 0.5
	III-IV	10 ± 1.2	15 ± 1.6	15 ± 1.6
	V-VI	13 ± 1.5	14 ± 2.1	12 ± 0.7
$\beta_{2/3}$	I-II	19 ± 0.7	19 ± 0.6	19 ± 0.7
	III-IV	18 ± 1.0	21 ± 1.0	18 ± 1.2
	V-VI	19 ± 0.4	18 ± 0.5	20 ± 0.2

Mean (\pm S.E.M.) number of immunogold particles per 100 μm^2 of 70- μm sections taken from the frontoparietal somatosensory cortex of rats exposed to long-term treatment with either vehicle, diazepam or imidazenil. Statistical significance was determined by analysis of variance followed by Fisher's Least Significant Difference test (** $p < 0.01$).

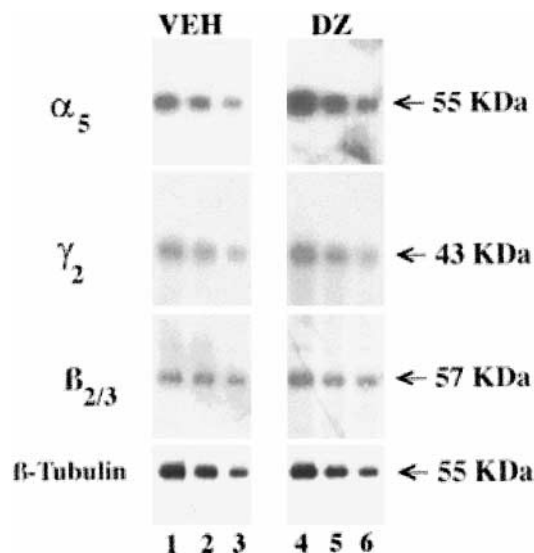


Fig. 4. Computerized scan of Western blots illustrating the levels of the GABA_A receptor α_5 , γ_2 and $\beta_{2/3}$ subunits in crude synaptic membranes prepared from the frontoparietal motor cortex of rats that received long-term treatment with either vehicle (left) or diazepam (right). The lower blots of the housekeeping protein β -tubulin demonstrate equivalent loading of the three different concentrations of protein: 12 μg (lanes 1 and 4), 8 μg (lanes 2 and 5) and 4 μg (lanes 3 and 6). Note the apparent increase in immunoreactivity for the α_5 , γ_2 and $\beta_{2/3}$ subunits of the GABA_A receptor in diazepam-tolerant rats.

appeared to be higher in diazepam-treated than in vehicle-treated rats. The diazepam-induced increase in immunolabelling of these three GABA_A receptor

subunits observed in this western blot analysis is in accordance with the changes in labelling densities observed immunocytochemically. While the western blot analysis was not quantified, as it was primarily intended to be qualitative in nature, these data provide further evidence that tolerance to the anti-convulsant effect of diazepam is associated with an increase in the expression of the α_5 , γ_2 and $\beta_{2/3}$ subunits of the GABA_A receptor in the FrPaM cortex of rats.

DISCUSSION

The use of immunogold quantitative technology utilizing antibodies specific for the α_1 , α_2 , α_3 , α_5 , γ_2 and $\beta_{2/3}$ subunits of the GABA_A receptor, has allowed us to produce evidence that long-term treatment with diazepam, using a dosage schedule that produces anticonvulsant tolerance,³⁰ changes the expression densities of the α_1 , α_3 , α_5 , γ_2 and $\beta_{2/3}$ subunits in the FrPaM and the FrPaSS cortices. Our results also demonstrate that analogous long-term treatment with the PAM imidazenil, which also occupies the receptors with high affinity but unlike diazepam has a uniformly low intrinsic activity in several GABA_A receptor subtypes, does not produce tolerance and is not associated with similar changes.

To address the question of changes in GABA_A receptor subunit expression following benzodiazepine tolerance, the immunogold labelling technique was selected over the more conventional immunostaining technique because the former offers the advantage of allowing a more accurate quantitative comparison of the expression density of any given GABA_A receptor subunit. Moreover, the distinct laminar distribution of the individual subunits revealed by immunogold labelling in this experiment (see Fig. 1) was similar to the distribution reported using the same antibodies with more conventional colorimetric staining:¹⁹ the α_1 , $\beta_{2/3}$ and γ_2 subunits are most intensely labelled in layers I, III-IV and VI, with only weak labelling in layer V. In contrast, the α_5 subunit is most pronounced in layers V and VI, with the lowest labelling occurring in layers II-IV. These subunits are characterized by diffuse labelling of the neuropil (see Figs 1 and 3), with labelling of some individual neuronal cell bodies distinguishable at higher magnifications. As previously discussed, the abundant expression of various GABA_A receptor subunits in the neocortex necessitates the use of subsaturating concentrations of antibody. This gold immunolabelling technique therefore cannot be used to make comparisons of subunit expression densities among different subunits. We have, however, shown that this immunogold technique is suitable for comparing the expression density of a particular subunit in discrete brain areas of animals that have been exposed to different drug treatments (Tables 2 and 3).

The most striking change in GABA_A receptor subunits that can be related to tolerance to the

anticonvulsant effects of diazepam is a 37% decrease in the α_1 subunit and a 150% increase in the α_5 subunit in layers III–IV of the FrPaM cortex. These diazepam-induced changes in subunit expression were substantiated by western blot analysis to reflect expression of the full subunit molecule. Whether a subunit such as α_1 is actually being replaced by another subunit such as α_5 in the assembly of one or more GABA_A receptor subtypes, cannot currently be indicated by these or other studies because of the lack of appropriate methodology to study the GABA_A receptor subunit assembly. Immunoprecipitation studies have reported, however, that the α_1 subunit is several-fold more abundant than the α_5 subunit in the rat neocortex,^{5,41} suggesting that the decrease in the number of α_1 subunit molecules in the FrPaM cortex of diazepam-tolerant rats detected with our immunogold technique is comparable in size with the larger increase in the α_5 subunit density found in this same brain area. Diazepam has been shown to have greater intrinsic modulatory activity in those GABA_A receptors including the α_1 subunit than in those assembled with the α_5 subunit.^{15,34,56} Given the magnitude and the direction of the changes in the expression of these subunits, it is possible to propose that a long-term *in vivo* exposure to a FAM such as diazepam changes the expression of GABA_A receptor subtypes by decreasing the density of receptors that include the α_1 subunit and by increasing that of receptor subtypes that include the α_5 subunit in select brain areas. The changes in GABA_A receptor subunit expression associated with diazepam tolerance perhaps reflect the operation of a unique homeostatic mechanism directed at maintaining the regulation of GABA-gated Cl⁻ current intensities within the limits of certain physiological requirements. A modification of this putative regulatory mechanism as a substrate for benzodiazepine tolerance is in keeping with the down-regulation of GABA-elicited Cl⁻ currents reported to occur during benzodiazepine tolerance.²¹ It is further supported by the fact that PAMs such as imidazenil, which bind to the benzodiazepine receptors with high affinity but only produce a modest amplification of GABA-elicited Cl⁻ currents,²⁴ can maintain unaltered their pharmacological profile following long-term treatment with progressively increasing doses.^{1,23,45}

Previous studies in our laboratory showed that 14-day treatment with diazepam identical to that used in these studies did not modify GABA_A receptor binding density or affinity in brain tissue homogenates.³⁰ Since the presence of the γ subunit is necessary for the binding of benzodiazepines to the GABA_A receptor,²⁵ this lack of a change in binding, despite the increased expression of the γ_2 subunits, indicates that the increase in γ subunit expression in diazepam-tolerant rats is not very likely to be responsible for either the down-regulation of the GABA_A receptor function or the tolerance to benzodiazepine action. Presently, one cannot establish the subunit stoichi-

ometry of the GABA_A receptor expressed in normal or diazepam-tolerant rats, and thus we cannot rule out the possibility that the increase in γ_2 subunits is associated with a substitution of α_1 subunits in some receptors that contain two such subunits. The increase in β_2 or β_3 protein expression may similarly not be critical to the onset of benzodiazepine tolerance since the β subunits do not appear to affect substantially the benzodiazepine pharmacology profile.^{15,26,55} Furthermore, the increase in the expression of the γ_2 and $\beta_{2/3}$ subunits was not paralleled by an increase in the expression of their respective mRNAs.³⁰ These results, taken together with the demonstration that during diazepam tolerance the expression of mRNA for the α_1 and the α_5 subunits of the GABA_A receptor changes in the same direction as that of their translation products,³⁰ provide support for the notion that tolerance to FAM benzodiazepines may actually be the consequence of changes in the molecular form of the α subunit included in certain GABA_A receptor subtypes.

A caveat that still requires discussion, however, is that the changes in subunit expression observed in the present immunohistochemical experiments may reflect changes in subunits expressed in a cytosolic compartment and therefore may not have a direct functional role. There are various pools of GABA_A receptor subunits in the brain,^{8,14} which are located both in glial and neuronal membrane, and in cytosol.⁷ Some of the cytosolic pools are likely to reflect a metabolic process, while some of those located in neuronal membranes may be operative in synaptic signal transduction. Unfortunately, these latter functional pools cannot be differentiated in our histochemical studies from the metabolic pools. Future research directed at determining changes occurring exclusively in the membrane is therefore necessary to determine the exact topology of the changes detected in these studies. The use of cultured cells dissociated from brain areas where tolerance-induced changes in subunit expression are observed, combined with single and double immunolabelling of freeze-fractured replicas, will enable determination of whether the changes in subunit expression observed in these studies reflect changes in the structure of neuronal membrane-bound GABA_A receptors.

In this study, the investigation was focused on the FrPaM and the FrPaSS cortices because previous studies in our laboratory showed that diazepam-induced changes in the expression of mRNA encoding for GABA_A receptor subunits were different in these two cortical areas despite the known similarities in both their cellular organization and the expression of GABA_A receptor subunit mRNAs and proteins. The FrPaSS cortex is the primary receiving area for somatosensory information from the thalamus and has efferent projections to the FrPaM cortex, which is predominantly involved in the mediation of motor activity.³¹ Although the brain areas involved in the mediation of the anticonvulsant effects of benzo-

diazepines have not been well defined, based on their functional distinction, the FrPaM cortex might be expected to play a more significant role than the FrPaSS cortex in the mediation of the anticonvulsant effects of diazepam. The results of these studies confirm that changes in GABA_A receptor subunit expression following tolerance to the anticonvulsant effects of diazepam are different and more dramatic in the FrPaM cortex than in the FrPaSS cortex. Moreover, the most significant changes in subunit expression occurred in layers III–IV of the FrPaM cortex, where the majority of GABAergic synaptic contacts are made.³ In these cortical layers, GABAergic neurons exert powerful inhibitory control over thalamic inputs, shape functional properties of spiny stellate cells, and modify cortical output through descending pyramidal neurons.^{36,58,62} The diazepam-elicited changes in the strength of GABAergic inhibition and disinhibition in the middle layers of this cortical circuitry may produce important changes in columnary pyramidal cell association and synchronous firing. Prolonged changes in GABAergic tone at this level may trigger tolerance to benzodiazepines by changing regulatory mechanisms attending to the expression of subunits in the GABA_A receptor structure in an attempt to re-establish normal physiological regulation by the GABAergic system.

CONCLUSIONS

The changes in the expression of the GABA_A receptor subunit molecular forms revealed in this study to be associated with benzodiazepine tolerance, combined with our previous findings of similar changes in the expression of their mRNA, suggest that tolerance following long-term exposure to diazepam may be the result of changes in the subunit composition, or more specifically, in the α subunit composition of the GABA_A receptors in discrete regions of the frontoparietal cortex. We believe that this may not be the only site in which changes in GABA_A receptor structure occur during diazepam

tolerance. It should be noted that just as protection against convulsions is only one of the many pharmacological properties of diazepam, the frontoparietal cortex is only one of the many brain areas that are rich in GABA_A receptors. It is possible therefore that the different pharmacological effects of benzodiazepines are a result of their activity in different brain regions, and in turn tolerance to these different pharmacological effects may be the result of changes in GABA_A receptor composition in these respective areas. From the results of this study it appears that benzodiazepines may produce their anticonvulsant effects by potentiating GABAergic activity in select regions of the frontoparietal cortex. Similarly, benzodiazepines may produce their anxiolytic effects by stimulating GABA_A receptors in limbic structures such as the amygdala and the septum that have been implicated in the modulation of anxiety.^{52,53} Their amnesic effects may result from their action on GABAergic circuits in the hippocampus and the limbic cortex,^{37,59} while their ataxic effects may be due to their activity in the striatum, the cerebellum or the spinal cord.³⁵ These possibilities point to the necessity for future studies that would examine the relationship between the development of tolerance to these pharmacological properties of benzodiazepines and the onset of changes in the subunit assembly of the GABA_A receptor in different brain areas. Elucidation of these relationships, together with the definition of methods to study the quaternary structure of the GABA_A receptor subunits in the neurons of different brain areas, could lead to a better understanding of the diversity of GABA_A receptor subunit assembly and provide insights into the molecular mechanism underlying tolerance to benzodiazepines in different brain regions.

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