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Modifications of γ-Aminobutyric Acid$_A$ Receptor Subunit Expression in Rat Neocortex during Tolerance to Diazepam

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SUMMARY

We evaluated whether tolerance to the antagonism of bicuculline-induced seizures by diazepam is associated with changes (i) in the content of mRNAs encoding for γ-aminobutyric acid$_A$ (GABA$_A$) receptor subunits, (ii) in the expression density of these subunits, and (iii) in the 1,4-benzodiazepine binding site characteristics in discrete neocortical structures. We found that in diazepam-tolerant rats, the content of the mRNA encoding for the α1 subunit of the GABA$_A$ receptor decreased in the frontoparietal motor (FrPaM) cortex and in the hippocampus (42% and 20%, respectively) but not in the frontoparietal somatosensory (FrPaSS) cortex, striatum, olfactory bulb, and cerebellum. In the FrPaM cortex, γ2S and γ2L subunit mRNA contents were also decreased (48% and 30%, respectively), whereas that of α5 was increased (30%). In the FrPaM and FrPaSS cortices as well as in cerebellum of diazepam-tolerant rats, the content of α2, α3, α6, β2, and δ subunit mRNA was unchanged, as was the content of α2, α5, γ1, and γ2S subunit mRNA in the hippocampus. Furthermore, the reduction in α1 subunit mRNA content in the FrPaM cortex and the anticonvulsant tolerance to diazepam returned to control values 72 hr after termination of the protracted diazepam treatment. Rats receiving a treatment with imidazenil in doses equipotent and with a schedule identical to that of diazepam failed to exhibit tolerance to the anticonvulsant action of this drug or cross-tolerance to diazepam. In these rats, the content of mRNA encoding for α1, α2, α3, α5, α6, γ1, γ2S, γ2L, and δ GABA$_A$ receptor subunits failed to change in the FrPaM and FrPaSS cortices, in the hippocampus, and in the other brain areas that were studied in diazepam-tolerant rats. Although the density and affinity of [^{3}H]flumazenil and [^{3}H]imidazenil binding failed to change in the FrPaM and FrPaSS cortices of diazepam-tolerant rats, the expression density of α1 subunit immunogold labeling decreased by 37%, whereas that of α5, γ2L/S, and β2/3 increased by 158%, 50%, and 47%, respectively, in the FrPaM cortex, and the density of the α5 subunit selectively increased (209%) in the FrPaSS cortex. In contrast, the immunogold labeling density of the α1, α5, γ2L/S, and β2/3 subunits failed to change in either the FrPaM or FrPaSS cortex of rats receiving protracted imidazenil treatment.

Rodents receiving repeated, long term treatment with diazepam, a sedative, anxiolytic, and anticonvulsant BZ acting with high intrinsic activity as a positive allosteric modulator of GABA action on selected native and recombinant GABA$_A$ receptor subtypes [classified as a selective allosteric modulator (1)], exhibit a tolerance to its sedative and anticonvulsant actions (2–5). In contrast, a similar treatment with equipotent doses of imidazenil, a nonsedative, anxiolytic, and anticonvulsant BZ (6, 7), acting as a partial allosteric modulator of GABA action at a large number of native and recombinant GABA$_A$ receptor subtypes [classified as a partial allosteric modulator (1, 6)], fails to induce anticonvulsant tolerance (4, 8).

The tolerance to the anticonvulsant action of diazepam appears to be associated with a down-regulation of GABA$_A$ receptor function (9, 10). This suggestion is compatible with the inability of diazepam to potentiate neuronal responses in various brain areas of diazepam-tolerant rats to the electrophoretic application of GABA (9, 10), with a reduction in the coupling efficiency of GABA/BZ-binding measured *ex vivo* in synaptic membranes obtained from different brain regions of diazepam-tolerant rats (11), and with the sensitization to the convulsant effects of negative allosteric modulators of GABA action (i.e., β-carbolines) in mice receiving protracted treatment with diazepam or other BZs endowed with high intrinsic activity (12). However, the molecular nature of this down-regulation remains unclear.

GABA$_A$ receptors are hetero-oligomeric intrinsic membrane proteins assembled with five subunits derived from four subunit families (α, β, γ, and δ) that are encoded by ≥15 different genes (13, 14). More than 800 structurally different GABA$_A$ receptors are theoretically compatible with the vari-

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ABBREVIATIONS: BZ, 1,4-benzodiazepine; GABA, γ-aminobutyric acid; FrPaM, frontoparietal motor; FrPaSS, frontoparietal somatosensory; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

ety of subunit mRNA repertoires detected in various brain structures (15). Electrophysiological studies with a number of recombinantly expressed GABA$_A$ receptor subtypes have demonstrated that GABA potency and BZ modulatory efficacy vary at structurally different GABA$_A$ receptors and depend on the stoichiometry of the α, β, γ, and δ subunit variants participating in the receptor assembly (16–18).

Although there have been some reports indicating that tolerance to BZs is associated with a modest reduction in the number of BZ binding sites and γ2 subunit mRNA expression (19–22), contradictory reports have appeared (23–25). In these latter reports, the GABA$_A$ receptor down-regulation associated with the development of tolerance to BZs after long term treatment (23–25), in the absence of pronounced changes in the affinity or density of BZ binding sites, may be due to changes in the subunit composition of the receptor complex. In support of such a hypothesis, studies that have examined the expression density of mRNAs encoding for the α1 and γ2 GABA$_A$ receptor subunits have generally reported selective decreases in these subunits in the cortex and/or hippocampus of rats and mice treated chronically with highly efficacious BZs (22, 26–30). However, conclusions cannot be drawn concerning the identity of subunit types within the pentameric structure of GABA$_A$ receptors because a marginal decrease (10–30%) in the content of α1 and γ2 subunit mRNAs, without changes in α5 and β1 subunit mRNA content, was detected in the entire neocortex of brains obtained from BZ-tolerant rats or mice in some of these studies (27, 28) but not in others (22, 30). It is therefore unlikely that such small and inconsistent decreases in the content of α1 and γ2 subunit mRNA can explain the marked tolerance that develops to the anticonvulsant action of diazepam and the concomitant functional GABA$_A$ receptor down-regulation. Although there is no published comprehensive analysis of the GABA$_A$ receptor subunits in different brain areas of rats tolerant to diazepam, it seems likely that the changes in subunit mRNA content observed in neocortex or hippocampus of diazepam-tolerant rats reflect a larger alteration in GABA$_A$ receptor subunit expression restricted to discrete functionally distinct neocortical areas. To test this hypothesis, we determined the affinity and density of BZ recognition sites, the content of several mRNAs encoding for different GABA$_A$ receptor subunits, and the expression density of their respective translation products in different areas of the FrPaM and FrPaSS cortices, hippocampus, cerebellum, striatum, and olfactory bulb of rats made tolerant to the antagonistic action of diazepam against bicuculline-induced seizures. We compared these results with those obtained in the same brain areas of rats exposed to a similar protracted treatment with equipotent anticonvulsant doses of imidazenil because these rats fail to develop tolerance to its antibicuculline action.

Experimental Procedures

Materials. Male Sprague-Dawley rats weighing 125–135 g (Zivic Miller, Pittsburgh, PA) were housed three per cage and maintained on a 12-hr light/dark cycle. Diazepam and imidazenil were obtained from Hoffman-La Roche (Nutley, NJ).

Reagents used for oligodeoxynucleotide synthesis were purchased from Applied Biosystems (Foster City, CA) for use with the model 381 DNA synthesizer (Applied Biosystems). The enzyme Hot Tab DNA polymerase and [^{32}-P]dCTP (3000 Ci/mmol) were purchased from Amersham (Arlington Heights, IL), [^{3}H]Flumazenil was purchased from NEN (Boston, MA), and [^{3}H]imidazenil was obtained from Hoffman-La Roche. Random hexamers and dNTPs were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). The restriction enzyme *Bgl*II, the Moloney murine leukemia virus reverse transcriptase, guanidine isothiocyanate, CsCl, and agarose were purchased from GIBCO-BRL (Grand Island, NY).

Schedule for long term drug treatment. Equipotent amounts of diazepam or imidazenil (4) were suspended in water containing 0.05% Tween-20 and administered in 2 ml volume by oral gavage three times daily (at approximately 9:00 a.m., 2:00 p.m., and 7:00 p.m.) for 14 days at increasing doses (diazepam: days 1–3, 17.6 μmol/kg; days 4–6, 35.2 μmol/kg; days 7–10, 52.8 μmol/kg; and days 11–14, 70.4 μmol/kg; imidazenil: days 1–3, 2.5 μmol/kg; days 4–6, 5.0 μmol/kg; days 7–10, 7.5 μmol/kg; and days 11–14, 10 μmol/kg). Control rats received only vehicle.

Bicuculline convulsion test. The threshold for bicuculline convulsion was determined by infusing a solution of (+)-bicuculline (0.27 mM) into the tail vein of unrestrained rats at constant rate (0.46 ml/min) with a Sage infusion pump (model 341, Orion, Cambridge, MA). The convulsive threshold dose was defined as the dose required to elicit the first visual sign of tonic-clonic convulsion.

Anticonvulsant tolerance test. Rats receiving long term diazepam or imidazenil treatment were left drug free for 24 hr before receiving an equipotent standard oral dose of either diazepam (17.6 μmol/kg) or imidazenil (2.5 μmol/kg). The administration of diazepam or imidazenil was followed 30 min later by the intravenous infusion of bicuculline (convulsion test) (4).

Brain dissection. Immediately after decapitation, the olfactory bulbs were removed and stored at −70°. With a Jacobovitz brain slicer (Zivic Miller), brains were cut into 1-mm-thick slices. The slices obtained at 0.2–3.2 mm anterior to bregma (31) were mounted on a coverslip at −4°, and disks (1.5-mm diameter) were punched out from the striatum and the FrPaM and FrPaSS cortices. Similarly, the slices obtained at 3.8–5.8 mm and 10–12 mm posterior to bregma were used to punch out disks (1.5-mm diameter) from the hippocampus and cerebellum, respectively. Discs from each area were pooled together and used for RNA isolation or radioligand binding studies.

RNA isolation and quantitative RT-PCR analysis. Total RNA was isolated from the frozen tissue by homogenization in 5 M guanidine isothiocyanate/5.7 M CsCl and ultracentrifugation as previously described (32). The yield of total RNA was determined by measuring the absorbance of an aliquot of the ethanol-precipitated stock at 260/280 nm. Quantitative RT-PCR analysis was performed as previously described (33). Briefly, known and increasing amounts of cRNA derived from the appropriate standard template [prepared as described by Grayson *et al.* (34)], ranging from 300 to 400 base pairs (33), were added to a constant amount (1 μg) of total RNA. The RNA/cRNA mixtures were denaturated for 5 min at 80° and then reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase in the presence of 1 mM dNTPs and 2.5 mM random hexamers in a total volume of 20 μl. The reaction was carried out by incubating the mixture for 1 hr at 37° after a 10-min preincubation at room temperature. The reverse-transcribed material was then amplified with Hot Tab DNA polymerase in a Thermal Cycler (Perkin-Elmer). Before amplification, the cDNA was heated at 98° for 5 min and quickly chilled on ice to denature the starting template. The amplification reaction was performed in a 100-μl volume containing 200-μM concentration of dNTPs, 2.5 units of Hot Tab Polymerase, and 1 μM concentration of specific subunit primers: α1 subunit, 5′-AGCTATACCCCTAACTTAGCCAGG-3′ and 5′-AGAAAGCGATTCTCAGTGCAGAGG-3′; α2 subunit, 5′-ACAAGAAGCCAGAGAACAAGCCAG-3′ and 5′-GAGGTCTACTGGTAAGCTCTACCA-3′; α3 subunit, 5′-CAACATAGTGGGAACCACCTATCC-3′ and 5′-GGGGTTTG GGATTTTGGATGCTTC-3′; α5 subunit, 5′-CAAGAAGGCCTTGGAAGCAGCTAA-3′ and 5′-GGTTTCCTGTCTTACTTTGGAGAG-3′; α6 subunit, 5′-AAGCCCCCGGTAGCAAAGTCAAAAA-3′ and 5′-TTCCTGGCTGCAAACTACTCGACA-3′; β2 subunit, 5′-TGAGATGGCCACATCAGAAGCAGT-3′ and 5′-TCATGGGAGGCT-

GGAGTTTAGTTC-3′; γ1 subunit, 5′-C AGAGACAGGAAGCT-GAAAAGCAAA-3′ and 5′-CGAAGTGATTATATTGGACTAAGCC-3′; γ2S subunit, 5′-AAGAAAAACCCTGCCCCTACAATT-3′ and 5′-TTCGTGAGATTCAGCGA ATAAGAC-3′; γ2L subunit, 5′-CTTCTTCG-GATGTTTTCCTTCAAG-3′ and 5′-CATAGGGTATTAGATCGTTG-GACT-3′; and δ subunit, 5′-TGAGGAACGCCATTGTCCTCTTCT-3′ and 5′-ACCACCGCACGTGGTACATGTAAA-3′ (33, 34). Trace amounts of [^{32}P]dCTP were added to the reaction for subsequent quantification. Amplification products from both mRNA and cRNA templates, in a postamplification step, were digested with *Bgl*II, and the products were separated through agarose gel electrophoresis (33). The ethidium bromide-stained DNA bands were excised and counted for isotope incorporation. The counts incorporated into the reverse-transcribed and amplified standard cRNA divided by the counts incorporated into the corresponding subunit amplification product were plotted as a function of the known amount of internal standard cRNA added to the test sample. Absolute quantities were determined from the point of equivalence (i.e., the point at which the ratio of counts is unity). The assay procedure reliability was verified by assaying three or four tissue samples in quadruplicate. Data were analyzed with the use of one-way analysis of variance. As expected, for all of the subunits studied, the intra-assay variation was lower than the interassay variation, as indicated by the smaller value for the mean squares (Table 1). Moreover, the ratio of the variances (*F* value) indicates that the variation between mean values of different samples did not reach significance at $\alpha = 0.05$ (Table 1).

Radioligand binding assay. Selected brain areas (see Tissue dissection) were frozen at −20° until the day of the experiment. Membranes were prepared from the frozen tissue through homogenization in 50 mM Na_2HPO_4, pH 7.4. The resulting homogenates were centrifuged at 20,000 × *g* for 10 min. The supernatants were discarded, and the pellets were resuspended in the above buffer to a final protein concentration of 1 mg/ml. The binding assay was carried out with 100 μg of membrane in a final volume of 250 μl in presence of various [^{3}H]BZ recognition-site ligands. Specific binding was defined as the difference between binding in the presence and in absence of 10 μM unlabeled diazepam.

Immunocytochemistry. Polyclonal antibodies directed against specific subunit peptides were used as primary antibodies [α1, residues 1–16 (35) raised in rabbit; α5, residues 1–10 (36), and γ2L/S, residues 1–29 (36) raised in guinea pig, as well as mouse monoclonal antibody bd-17 directed against an epitope common to both the β2 and β3 subunits of the $GABA_A$ receptor (37, 38)]. The specificity of the antibodies was tested immunohistochemically on label-fractured replicas of human embryonic kidney 293 cells transfected with cDNAs encoding the various $GABA_A$ receptor subunits (39). All of the antibodies were provided by J. M. Fritschy (Institute of Pharmacology, University of Zurich, Zurich, Switzerland), except the antibody for the β-chain of the $GABA_A$ receptor, which was purchased from Boehringer Mannheim (Indianapolis, IN) (for information concerning the further characterization of the specificity of these antibodies, see Refs. 1, 36, 39, and 40). Goat anti-rabbit, goat anti-guinea pig, and goat anti-mouse secondary antibodies conjugated to colloidal 1-nm gold particles were purchased from Goldmark Biologicals (Phillipsburg, NJ).

Rats were anesthetized with equithesin (43 ml of H_2O, 425 g of chloral hydrate, 1.25 g of Mg SO_4, 26.6 ml of propylene glycol, 12.5 ml of ethanol, 18 ml of Nembutal) (3 ml/kg) and perfused intracardially with 100 ml of PBS, followed by 100 ml of fixative (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M PBS, pH 7.2). Brains were removed and kept overnight in fixative and then embedded in PBS containing 30% sucrose for 3 days at 4°. Brains were frozen, and 70-μm coronal sections from the FrPaM and FrPaSS cortices were cut with a cryostat, maintained in PBS for 2 days at 4° to remove the embedded sucrose, and incubated at room temperature in RPMI 1640 (GIBCO) for 30 min followed by 30 min in 1% BSA in PBS. The sections were then incubated overnight at 4° followed by 2 hr at room temperature with the primary antibody diluted in 1% BSA in PBS (α1, 1:20,000; α5, 1:3,000; γ2, 1:5,000; and β2/3, 1 μg/ml), rinsed twice for 30 min in 1% BSA, and then incubated for 60 min at room temperature with the corresponding gold-labeled secondary antibody [goat anti-rabbit IgG (α1), goat anti-guinea pig IgG (α5 and γ2L/S), and goat anti-mouse IgG (β2/3)]. Sections were then rinsed four times for 30 min in 1% BSA in PBS and treated in the dark with a silver-enhancing solution (Goldmark Biologicals) for 20 min. After several rinses with distilled water, the sections were collected onto microscope slides, air-dried, counterstained with toluidine blue, and photographed with a camera mounted to an Olympus BH-2 microscope. An identical protocol, except that 1% BSA in PBS was substituted for the primary antibody, was followed for the control sections from which nonspecific labeling was determined. Typical photomicrographs of total and nonspecific immunogold labeling for α1, β2/3, and γ2L/S are depicted in Fig. 1.

Such an approach provides an opportunity to carry out comparisons of the immunoreactivity of a specific antibody in various brain structures. However, to make accurate comparisons of gold particle densities, the immunoreactions were carried out with subsaturation concentrations of antibodies. Therefore, for each subunit, we compared the relative immunoparticle density of a given antibody across the three drug conditions in a given brain area, rather than comparing the labeling density in a given area across the different subunits. Thus, we attempted to minimize possible errors due to the different affinities of the antibodies specific for each of the subunits studied.

Results

Anticonvulsant efficacy of diazepam and imidazenil after long term treatment. In rats treated three times daily for 14 days with diazepam or equipotent doses of imidazenil, we estimated the degree of tolerance to the anticonvulsant action of each drug by measuring the threshold dose of bicuculline necessary to elicit convulsions.

In naive rats, an equipotent standard dose of diazepam (17.6 μmol/kg) or imidazenil (2.5 μmol/kg) administered by oral gavage 30 min before the bicuculline challenge increased

TABLE 1

Characteristics of the quantitative RT-PCR assay for α1, β2, and γ2S $GABA_A$ receptor subunits

$GABA_A$ receptor subunit	Mean value[a]	Source of variation	Sum of squares	Degree of freedom	Mean squares	F^b
α1	935	Interassay	82,861	3	27,620	2.25
		Intra-assay	147,344	12	12,278	$F_{(95\%)} = 3.49$
						$F_{(99\%)} = 5.95$
β2	208	Interassay	766	2	383	1.43
		Intra-assay	2,413	9	268	$F_{(95\%)} = 4.26$
						$F_{(99\%)} = 8.02$
γ2S	190	Interassay	6,723	3	2,241	2.26
		Intra-assay	11,926	12	993	$F_{(95\%)} = 3.49$
						$F_{(99\%)} = 5.95$

[a] Mean of three or four samples, each assayed in quadruplicate.
[b] Differences between the mean values were not significant at $\alpha = 0.05$.

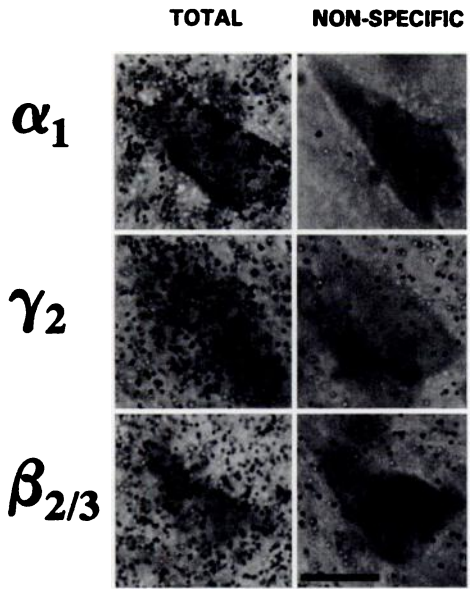

Fig. 1. Photomicrograph of both total and nonspecific immunogold labeling of the α1, γ2S/L, and β2/3 subunits of the GABA$_A$ receptor through the FrPaM cortex of normal rats. The total labeling represents the specific as well as nonspecific binding observed after incubation with both the primary and secondary antibody (see Experimental Procedures for protocol), whereas nonspecific labeling is observed in the control sections that were incubated with only secondary antibody (α1, goat anti-rabbit; γ2S/L, goat anti-guinea pig; β2/3, goat anti-mouse). In the total sections, note the heightened concentration of gold particles (labeled subunits) on the soma of pyramidal cells. In the nonspecific sections, note that the goat-anti-rabbit secondary antibody seems to produce the least nonspecific binding. *Calibration bar*, 10 μm.

the convulsive threshold dose of bicuculline by ~3-fold (Table 2). After 14 days of diazepam treatment (see Experimental Procedures), the standard oral dose of diazepam failed to modify the threshold dose of bicuculline that elicits convulsions (Table 2).

In contrast, after 14 days of treatment with equipotent doses of imidazenil, the anticonvulsant activity of a standard oral dose of imidazenil or diazepam given 24 hr after the last dose of the protracted imidazenil treatment still increased the threshold dose of bicuculline by an extent identical to that of naive rats (Table 2). It is important to note that a standard anticonvulsant dose of imidazenil given to diazepam-tolerant rats still increased the threshold dose of bicuculline by an extent virtually identical to that observed in vehicle-treated rats (Table 2).

TABLE 2

Anticonvulsant efficacy of diazepam and imidazenil in rats receiving 14 days of treatment with vehicle, diazepam, or imidazenil

Long term treatment	Acute challenge 24 hours after termination of long term treatment[a]		
	Bicuculline threshold		
	Vehicle	Diazepam (17.6 μmol/kg)	Imidazenil (2.5 μmol/kg)
	μmol/kg		
Vehicle	1.3 ± 0.3	3.2 ± 0.3	3.3 ± 0.4
Diazepam	1.2 ± 0.2	1.4 ± 0.1[b]	3.4 ± 0.6
Imidazenil	1.2 ± 0.4	3.1 ± 0.5	3.4 ± 0.5

[a] The animals were treated for 14 days with vehicle, diazepam, or imidazenil (see Materials and Methods for doses and schedule) and were drug free for 24 hours before bicuculline test. At 30 min before bicuculline challenge, rats received a single oral dose of vehicle, diazepam, or imidazenil. Each value is the mean ± standard error for six rats.

[b] $p < 0.05$ compared with respective vehicle-treated rats (analysis of variance followed by Duncan's multiple-range test).

Radioligand binding assays. A series of binding experiments were performed to determine the affinity and density of BZ binding sites associated with GABA$_A$ receptors present in discrete brain regions of rats that for 14 days received diazepam, imidazenil, or vehicle and were without treatment during the 24 hr preceding the binding studies. Table 3 indicates that [^{3}H]flumazenil (2.5 nM) and [^{3}H]imidazenil (1.0 nM) binding densities in thoroughly washed, frozen, and thawed crude synaptic membranes prepared from different brain regions were not substantially modified by the long term treatment with diazepam or imidazenil. Although the small (10–15%) decrease in [^{3}H]flumazenil binding observed in the FrPaM cortex of diazepam-tolerant animals was significant at $\alpha = 0.05$ (Table 3), this decrease tended to disappear when the membranes were frozen, thawed, and washed repeatedly. Scatchard analysis reveals that the difference in [^{3}H]flumazenil binding found in the FrPaM cortex was due to a small decrease in affinity (K_d = 2.9 and 3.2 nM in vehicle and diazepam-treated animals, respectively) but not in B_{max} (1.2 and 1.1 pmol/mg protein in vehicle and diazepam-treated rats, respectively), suggesting that the decreased binding affinity of [^{3}H]flumazenil, when present, was very likely due to residual traces of diazepam still bound to the membrane preparations.

Quantitative RT-PCR determination of α1, α2, α3, α5, α6, β2, γ1, γ2S, γ2L, and δ GABA$_A$ receptor subunits mRNA content in brain of rats receiving long term treatment with diazepam or imidazenil. The quantitative profile of 10 GABA$_A$ receptor subunit mRNAs expressed in four brain areas of rats receiving vehicle, imidazenil, or diazepam for 14 days is shown in Fig. 2. Each brain structure has a characteristic subunit mRNA distribution profile. In the FrPaM and FrPaSS cortices (compare Fig. 2A with Fig. 2B) of rats receiving vehicle, the rank order of the subunit mRNA content was $\alpha1 \gg \alpha3 > \alpha2 > \beta2 > \alpha5 > \gamma2S > \gamma2L > \gamma1 > \delta > \alpha6$. The α6 transcript is expressed at very low levels, whereas that of the α1 subunit was by far the most abundant. In cerebellum, the content of the α1 subunit transcript was similar to that in the FrPaM and FrPaSS cortices, whereas that of α6 was highly expressed, and those of α2, α3, and α5 were barely detectable (Fig. 2C). In the hippocampus, the contents of α2 and α5 subunit mRNA were comparable to that of α1, which was lower in this structure than in the FrPaM and FrPaSS cortices, as well as in cerebellum (Fig. 2D). The content of the γ1 subunit transcript was greater in the FrPaM and FrPaSS cortices than in cerebellum, whereas the γ2S subunit mRNA content was higher in the hippocampus than in the FrPaM and FrPaSS cortices or cerebellum. The cerebellar content of the mRNA encoding for the δ subunit was greater than that in the FrPaM and FrPaSS cortices.

In rats receiving diazepam three times daily for 14 days, the absolute amount of α1 subunit mRNA decreased by 42% in FrPaM cortex and by 20% in hippocampus, but notably the α1 subunit mRNA content failed to decrease significantly in FrPaSS cortex or in cerebellum (Fig. 2). The α1 subunit transcript also failed to change in the olfactory bulb and striatum of rats receiving a protracted treatment with diazepam (Table 4). Fig. 2A shows that the α5 subunit mRNA content was

TABLE 3

[^{3}H]Flumazenil and [^{3}H]imidazenil binding to different brain regions of rats receiving long term treatment with vehicle, diazepam, or imidazenil

Long term treatment[a]	[^{3}H]Flumazenil				[^{3}H]Imidazenil	
	FrPaM cortex	FrPaSS cortex	Hippocampus	Cerebellum	FrPaM cortex	FrPaSS cortex
	fmol/mg of protein					
Vehicle	530 ± 20	510 ± 14	570 ± 30	390 ± 10	885 ± 48	990 ± 40
Diazepam	430 ± 14[b]	500 ± 8	600 ± 26	410 ± 30	820 ± 40	910 ± 30
Imidazenil	480 ± 20	495 ± 14			845 ± 65	850 ± 35

[a] Rats were treated for 14 days with vehicle, diazepam, or imidazenil with the schedule described in Materials and Methods. They were killed 24 hr after the last drug administration. Binding assay was performed in crude synaptic membranes prepared from 1.5-mm discs obtained from 1-mm slices (see text) with 2.5 nM [^{3}H]flumazenil and 1 nM [^{3}H]imidazenil. Each value is the mean ± standard error of five or six experiments, with each experiment was assayed in triplicate.
[b] $p < 0.05$ compared with vehicle-treated group (analysis of variance followed by Duncan's multiple-range test).

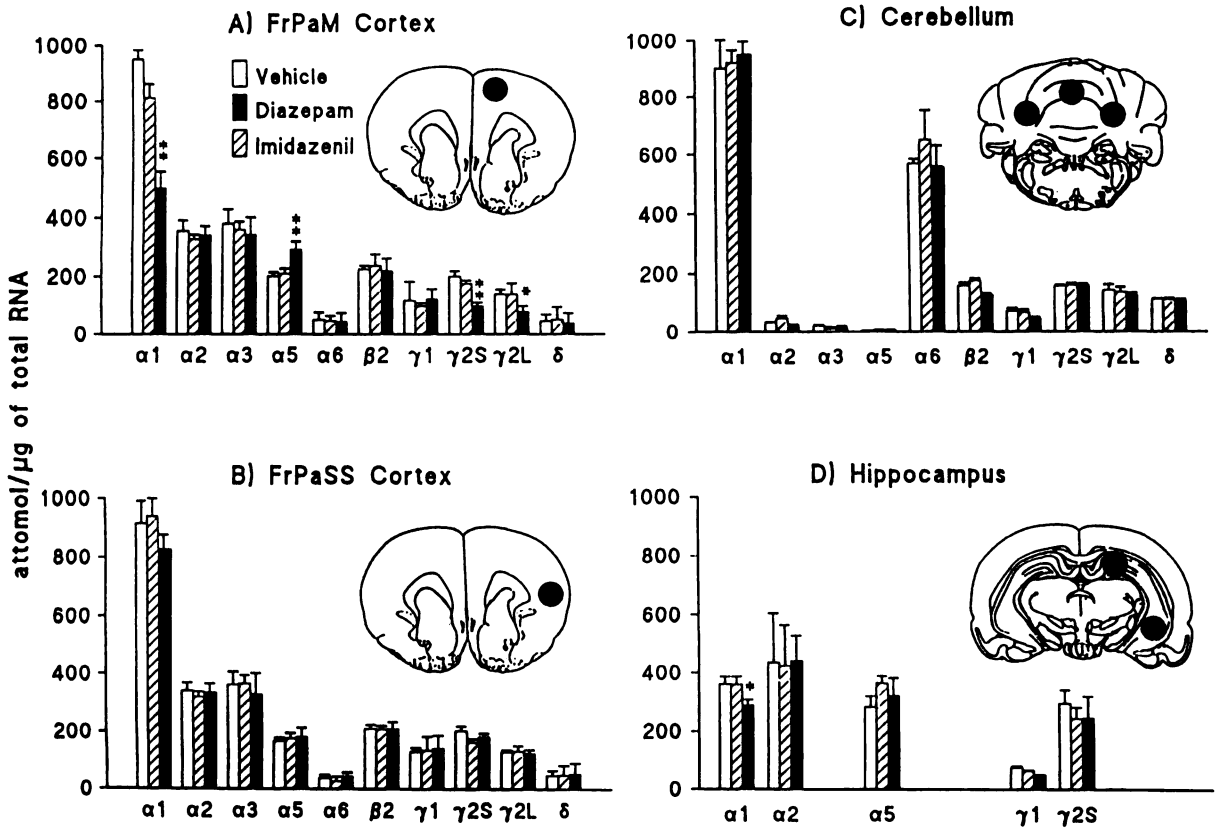

Fig. 2. Quantitative PCR analysis of molecular variants of α, β, γ, and δ $GABA_A$ receptor subunit mRNA content in different brain areas after long term treatment with vehicle, diazepam, or imidazenil. Rats were treated for 14 days with vehicle, diazepam, or imidazenil (see Experimental Procedures for regimen); they were killed 24 hr after the last drug administration. For each area analyzed, a schematic of the brain coronal section indicates the area from which the samples were obtained (●). In the hippocampus, the α3, α6, β2, γ2L, and δ subunit mRNA contents were not assayed. Data are mean ± standard error of five or six sets of competitive PCR experiments. Data were subjected to analysis of variance followed by Duncan's multiple-range test. *, $p < 0.05$ compared with respective vehicle-treated group. **, $p < 0.01$ compared with respective vehicle-treated group.

increased by ~30% in the FrPaM cortex but remained unchanged in the FrPaSS cortex, hippocampus, and cerebellum of diazepam-tolerant rats (Fig. 2, B–D); the levels of γ2L and γ2S subunit mRNA were decreased by ~50% and ~30%, respectively, in the FrPaM, but not in the FrPaSS cortex, cerebellum, or hippocampus of the same rats. The content of α2, α3, α6, β2, γ1, and δ $GABA_A$ receptor subunit mRNAs in both FrPaM and FrPaSS cortices, cerebellum, or hippocampus of diazepam-treated rats was virtually identical to that found in vehicle-treated rats (Fig. 2, A–D).

Importantly, the decrease in α1 subunit mRNA content detected in the FrPaM cortex of diazepam-tolerant rats returned to control levels by 72 hr after diazepam treatment discontinuation; at this time, the anticonvulsant tolerance also disappeared (Table 5). It should be noted, however, that in the experiment reported in Table 5, the content of other subunit mRNAs was not been determined. Importantly, in the brain areas of rats treated protractedly and repeatedly with imidazenil, the content of the mRNAs encoding for the $GABA_A$ receptor subunits that we analyzed was not significantly changed (Fig. 2, A–D, and Table 4).

Comparative immunohistochemical analysis of α1, α5, β2/3, and γ2 $GABA_A$ receptor subunits expression in rat FrPaM or FrPaSS cortices. Rats receiving a 14-day

TABLE 4

α1 mRNA content in different brain regions of rats receiving a long term treatment with vehicle, diazepam, or imidazenil

Brain region	α1 mRNA content Long term treatment[a]		
	Vehicle	Diazepam	Imidazenil
		attomol/μg of total RNA	
FrPaM cortex	950 ± 33	560 ± 55[b]	810 ± 49
FrPaSS cortex	910 ± 77	830 ± 49	980 ± 60
Hippocampus	360 ± 10	290 ± 16[c]	360 ± 27
Striatum	310 ± 45	280 ± 68	330 ± 24
Olfactory bulb	880 ± 39	890 ± 47	
Cerebellum	930 ± 130	970 ± 95	970 ± 100

[a] Drug administration schedule and sample collections were identical to those described in Table 2. Each value is the mean ± standard error of at least six animals (analysis of variance followed by Duncan's multiple-range test).
[b] $p < 0.01$ compared with vehicle-treated rats.
[c] $p < 0.05$ compared with vehicle-treated rats.

treatment with three daily doses of diazepam but not rats receiving the same treatment with equipotent anticonvulsant doses of imidazenil exhibited significant changes in the expression of the α1, α5, β2/3, and γ2L/S GABA$_A$ receptor subunits in the FrPaM cortex, as well as a change in α5 subunit expression in the FrPaSS cortex. More specifically, rats that were made tolerant to the anticonvulsant action of diazepam were found to have a 158% increase in the expression of the α5 subunit, a 50% increase in the expression of the γ2L/S subunit, a 47% increase in the expression of β2/3, and a 37% decrease in the expression of the α1 subunit in the FrPaM cortex, whereas in the FrPaSS cortex, there was a 209% increase in the expression of the α5 subunit, without changes in α1, β2/3, and γ2L/S subunit expression (Table 6).

Discussion

A previous study by Auta *et al.* (4) showed that rats receiving a 14-day treatment with three daily doses of diazepam (a BZ acting as a selective allosteric modulator at GABA$_A$ receptors) became tolerant to the antagonism of bicuculline-induced convulsions elicited by this BZ. Conversely, the same treatment schedule with equipotent anticonvulsant doses of imidazenil (a BZ acting as a partial allosteric modulator at GABA$_A$ receptors) failed to elicit such tolerance. In the current study, we confirmed these results and also showed a lack of cross-tolerance between imidazenil and diazepam. Thus, any inference or suggestion made on possible molecular mechanisms that explain the development of tolerance to diazepam must account for the lack of tolerance and cross-tolerance in rats receiving repeated administrations of equipotent doses of imidazenil.

In considering possible molecular mechanisms that might underlie diazepam tolerance, one can exclude the participation of changes in drug metabolism and disposition (4) and/or changes in the binding profile of the BZ recognition sites associated with GABA$_A$ receptors. In fact, as shown in Table 3, the binding profiles of [^{3}H]flumazenil and [^{3}H]imidazenil are virtually unchanged in different brain areas of rats tolerant to diazepam. One should not, however, overlook the remarkable changes in the expression of mRNA encoding for the GABA$_A$ receptor subunits in the neocortex and hippocampus of rats tolerant to diazepam as a possible mechanism underlying BZ tolerance. In the FrPaM neocortical area, there is a 42% decrease in the expression of the mRNA encoding for the α1 subunit; a 50% and 30% decrease in the γ2S and γ2L mRNA transcripts, respectively; and a 30% increase in the content of the mRNA encoding for the α5 subunit. Concomitantly in the hippocampus, there is a 20% decrease in the content of the mRNA encoding for the α1 subunit (Fig. 2). It is important to stress that these changes are very selective for the FrPaM cortex and hippocampus because no changes in the expression of these or the other six GABA$_A$ receptor subunit mRNAs (α2, α3, α6, γ1, β2, δ) were detected in the FrPaSS cortex, striatum, olfactory bulb, or cerebellum of diazepam-tolerant rats (Fig. 2 and Table 4).

As discussed above, in designing experiments that could provide circumstantial evidence in support of a correlation between altered GABA$_A$ receptor subunit mRNA content and diazepam tolerance, we contrasted mRNA data from rats tolerant to diazepam with those of rats receiving an equipotent treatment with imidazenil that fails to elicit tolerance to the anticonvulsant action of imidazenil or cross-tolerance to the anticonvulsant action of diazepam. Remarkably, we found that unlike brain areas obtained from rats receiving long term treatment with diazepam, none of the corresponding areas dissected from brains of rats receiving long term treatment with imidazenil showed changes in the content of mRNAs encoding for 10 different GABA$_A$ receptor subunits (Fig. 2). Furthermore, both the anticonvulsant tolerance and the altered expression of α1 GABA$_A$ receptor subunit mRNA in the FrPaM cortex of rats treated three times daily for 14 days with diazepam returned to normal levels by 72 hr after diazepam withdrawal (Table 5). These data suggest that the changes in mRNA content may be related to the presence of BZ tolerance. We cannot, however, conclude that they are the cause of the tolerance because the synchrony in the time dependency of the two independent variables has not yet been established.

Despite the strong similarity in the quantitative profile of GABA$_A$ receptor subunit mRNAs expressed in the FrPaM and in the FrPaSS cortices of naive rats (see Fig. 2), the various sensory and motor areas of the rat neocortex differ in the precision of peripheral mapping, cytoarchitecture, pattern of connectivity, type of physiological response that can be induced by electrical stimulation, and behavioral effect produced by specific lesions and discrete cortical electrical stimulations (41). Thus, the differences in the expression of mRNAs encoding various GABA$_A$ receptor subunits in FrPaM cortex, FrPaSS cortex, and hippocampus induced by protracted diazepam treatment suggest that the changes in the content of mRNAs encoding the α1, α5, and γ2 subunits do not result from a generalized nonspecific diazepam action on DNA transcription rates or on mRNA stability. Presumably, these changes might be related to some as yet unknown intrinsic feedback-regulatory mechanisms that control the flexibility in the expression of GABA$_A$ receptor subunit mRNA and in the receptor subunit assembly. This flexibility regulates GABAergic synaptic strength of selected neuronal circuits during adaptation to protract BZ treatment.

The data reported in the current study provide the first quantitative measurement of 10 specific mRNAs encoding for GABA$_A$ receptor subunits in several discrete brain regions of rats during a documented tolerance to diazepam. Previously, with the use of a semiquantitative method to measure the content of mRNA encoding for GABA$_A$ receptor subunits,

TABLE 5

Tolerance to the anticonvulsant effect of diazepam correlates with decreased α1 $GABA_A$ receptor subunit mRNA content in FrPaM cortex of rats receiving long term diazepam treatment

Long term treatment[a]	Bicuculline threshold dose		α1 $GABA_A$ mRNA	
	18 hr drug free	72 hr drug free	18 hr drug free	72 hr drug free
	μmol/kg		*attomol/μg of total RNA*	
Vehicle	3.2 ± 0.20	3.0 ± 0.080	920 ± 34	880 ± 49
Diazepam	1.5 ± 0.040[b]	2.8 ± 0.070	500 ± 55[b]	900 ± 18

[a] Rats were treated for 14 days with vehicle or diazepam (see Materials or Methods for doses and schedule) and left for 18 or 72 hr drug free before convulsion test or mRNA measurement. Thirty minutes before convulsion test, rats received a standard oral dose (17.6 μmol/kg) of diazepam or vehicle. Each value is the mean ± standard error of five or six rats.

[b] $p < 0.05$ compared with respective vehicle long term-treated rats (analysis of variance followed by Duncan's multiple-range test).

TABLE 6

Immunogold particle density of $GABA_A$ receptor subunits in rats FrPaM and FrPaSS cortex after long term treatment with vehicle, diazepam, or imidazenil

Rats receiving long-term treatment with vehicle, diazepam, or imidazenil (see methods for details) were anesthetized and then, after fixation, brains were dissected and prepared for immunogold labeling. Each value represents the mean ± standard error.

$GABA_A$ subunit	FrPaM cortex					FrPaSS cortex				
	Vehicle	Diazepam		Imidazenil		Vehicle	Diazepam		Imidazenil	
	Gold particles[a]	Gold particles[a]	Change[b]	Gold particles[a]	Change[b]	Gold particles[a]	Gold particles[a]	Change[b]	Gold particles[a]	Change[b]
			%		%			%		%
α1	27 ± 0.5	17 ± 2.0[c]	−37	23 ± 1.3	−15	27 ± 2.6	24 ± 1.6	−11	28 ± 2.9	+4
α5	12 ± 1.6	31 ± 1.5[c]	+158	15 ± 1.6	+25	11 ± 2.2	34 ± 2.3[c]	+209	13 ± 0.3	+18
γ2L/S	12 ± 1.3	18 ± 1.0[c]	+50	12 ± 0.6	0	14 ± 0.9	15 ± 1.6	+7	15 ± 0.8	+7
β2/3	19 ± 0.3	28 ± 0.7[c]	+47	18 ± 0.8	−5	19 ± 0.3	19 ± 0.6	0	19 ± 0.7	0

[a] No. of gold particles/100 μm^2.

[b] Percent changes in α1, α5, γ2, and β2/3 $GABA_A$ receptor subunit density were determined by comparing the mean number of gold particles/100 μm^2 in drug-treated groups with the mean number of gold particles counted in vehicle-treated rats, which were considered to be 100%.

[c] $p < 0.01$ compared with vehicle-treated controls (analysis of variance followed by Fisher's Least Significant Difference test).

Heninger *et al.* (26) and Primus and Gallager (28) demonstrated a decrease of 20–25% in α1 and a small decrease (10%) in γ2 subunit mRNA content in the neocortex of rats tolerant to diazepam. In our experiments, the decrease in α1 and γ2 subunit mRNA content detected in the FrPaM neocortex of diazepam-tolerant rats is significantly larger than that reported by Heninger *et al.* (26) and Primus and Gallager (28). However, in our study, the content of mRNA encoding for α1 and γ2 subunits was unchanged in the FrPaSS neocortex. Thus, it may well be that the difference between our results and those of Heninger *et al.* (26) and Primus and Gallager (28) relates to the size of the cortical sample used for the mRNA assay.

If BZ tolerance and functional down-regulation of $GABA_A$ receptors coincide with alterations in the expression of mRNAs encoding for specific $GABA_A$ receptor subunits in specific brain areas (Table 4 and Fig. 2), one should attempt to assess the consequences of these changes on the expression of the subunits, on the stoichiometry of the subunits assembly, and on the function of the specific $GABA_A$ receptor subtypes expressed in diazepam-tolerant rats. Unfortunately, the exact role played by each subunit in the structure and function of $GABA_A$ receptor subtypes remains unknown due to the lack of appropriate technology that would allow us to characterize the subunit stoichiometry and composition of various native $GABA_A$ receptors.

Most of what is known about the structure-activity relationship of $GABA_A$ receptor subtypes stems from several lines of research, including electrophysiological analysis of transiently expressed recombinant $GABA_A$ receptors (1, 16, 17, 42–44), *in situ* hybridization of mRNAs encoding for $GABA_A$ receptor subunits (45), $GABA_A$ receptor subunit identification in neurons through double and triple immunofluorescence staining with subunit-specific antibodies (36), and double immunolabeling of $GABA_A$ receptor subunits in freeze-fractured neuronal membranes of morphologically homogeneous primary cultures of cerebellar neurons prepared from neonatal rats (39, 40).

Each of these technologies, however, has its own intrinsic limitations. Electrophysiological studies with recombinantly expressed $GABA_A$ receptor subtypes indicate that their responsiveness to GABA and BZ depends on their characteristic subunit assembly. For example, the presence of α6 $GABA_A$ receptor subunit confers low susceptibility to the BZ amplification of GABA action but high GABA susceptibility (17, 18). The absence of a γ2 subunit abolishes the amplification of GABA action by BZ and reduces the channel-gating potency of GABA (17, 43, 44). However, we still have difficulties in extrapolating data obtained with recombinantly expressed receptors to native $GABA_A$ receptors. *In situ* hybridization studies of mRNA encoding for $GABA_A$ receptor subunits enables us to make predictions about which subunits might be used in the assembly of $GABA_A$ receptors in a given neuronal population (45, 46). However, the validity of these predictions awaits confirmation through the identification of the subunits expressed in neuronal membranes. In fact, the number of structurally different mRNA subunits expressed in a given neuronal population exceeds the number of subunits that can be used to form the pentameric $GABA_A$ receptor subtypes, and therefore the changes in the neuronal content of mRNA encoding $GABA_A$ receptor subunits may not necessarily predict changes in the steady state

levels of their respective translation products (33, 47). Therefore, as a first approach to elucidate the relationship existing between alterations in mRNA content and changes in the GABA$_A$ receptor structure and function, we decided to test whether there was any corresponding change in the amount of translation products expressed in the brain areas of rats tolerant to diazepam. We approached this problem histochemically, with immunogold labeling with antibodies specific for α1, α5, γ2, and β2/3 GABA$_A$ receptor subunits. We used the two adjacent FrPaM and FrPaSS neocortical areas because in animals tolerant to diazepam, we have detected changes in the content of specific mRNA encoding for α1, α5, and γ2 subunits of the GABA$_A$ receptor in the FrPaM, but not in the FrPaSS, cortex. In addition, the content of the mRNA encoding for the various GABA$_A$ receptor subunits was unchanged in both neocortical areas dissected from brain of rats receiving the protracted treatment with imidazenil.

It is known that in neocortical pyramidal cells and other types of neurons, the GABA$_A$ receptors are predominantly located in the somata and, in some cases, in the basal region of the apical dendrites and the axon hillock (36, 48). The neocortical pyramidal neurons are innervated by GABAergic basket cells, chandelier cells, and other GABAergic interneurons (41, 49–51). The rate of firing of the cortical GABAergic cells is faster than that of pyramidal cells (52). It is possible that their activity plays a role in mediating a time-dependent binding among populations of pyramidal cells located in different layers of the neocortex (53). This time-dependent binding might contribute to the characteristic columnar activity typical of neocortical pyramidal cell layers (54). However, with immunogold labeling, it is virtually impossible to differentiate which GABA$_A$ receptors are associated with glial cells and which are associated with neurons. Furthermore, it difficult to determine whether the GABA$_A$ receptor subunits labeled are actually present or are removed from the cell surface and sequestered into the cytosol (55) or assembled in vesicles (receptosomes) before being inserted into the neuronal membrane (36, 56). Ideally, the immunohistochemical and biochemical analyses should be carried out in single pyramidal cells isolated from brains of naive and BZ-tolerant rats with immunogold labeling in freeze-fractured membranes. In preparation for such experiments, we developed this technology in primary neuronal cultures of neonatal rat cerebellum using single and double immunolabeling to detect the coassembling of two specific GABA$_A$ receptor subunits in the same GABA$_A$ receptors (39, 40). Therefore, future experiments will include studying the relationship between changes of specific mRNA encoding for a given GABA$_A$ receptor subunit and the assembly of their translated products in GABA$_A$ receptor subtypes located in neuronal membranes with the use of dissociated pyramidal cells and immunogold labeling with different sizes of gold particles.

Cognizant of the inherent difficulties associated with the interpretation of gold immunolabeling experiments carried out in brain slices, we have begun to study whether there was any difference in the labeling intensity when the same antibody was used to label corresponding neocortical areas of brain of rats receiving a protracted treatment with diazepam, imidazenil, or vehicle.

Our data in Table 6 indicate that α1 subunit immunolabeling is decreased by ~37% in the FrPaM neocortical area but not in the corresponding neocortical area of rats receiving vehicle or imidazenil. In contrast, there was no change in α1 subunit immunogold-labeling intensity in the FrPaSS neocortical area of rats receiving vehicle, diazepam, or imidazenil. The immunogold-labeling particle density of the α5 subunit was markedly increased in both FrPaM and FrPaSS neocortical areas of rats receiving diazepam but failed to change in the corresponding areas of rats receiving imidazenil or vehicle. The immunolabeling intensities of γ2L/S and β2/3 subunits were increased selectively in the FrPaM neocortical area of rats receiving diazepam. However, they failed to increase in either area of rats receiving vehicle or imidazenil.

It is evident that the decrease in mRNA encoding for α1 GABA$_A$ receptor subunit is complemented very nicely by the immunogold-labeling data of the respective protein. Although there is a nice correlation between the increase in the mRNA encoding the α5 subunit and data on the increase of the respective protein in the FrPaM neocortical area of rats tolerant to diazepam, surprisingly, in this group of rats, the α5 subunit protein also increases in the FrPaSS cortex, and this increase occurs in absence of a change in the respective mRNA (compare Fig. 2 with Table 6). In rats receiving vehicle or imidazenil, the density of immunogold labeling for α5 subunit and that of the respective mRNA failed to increase in the two neocortical areas studied.

In the FrPaM cortex dissected from the brain of rats tolerant to diazepam, there was an increase in γ2L/S immunogold labeling with a significant decrease in the corresponding mRNA. Finally, in the FrPaM cortex, the mRNA content for the β2 subunit was not changed, but the protein expression of the β2/3 subunits was increased. Because the β2/3 subunit antibody was raised against the same β chain and recognizes both the β2 and β3 proteins and because the mRNA content of β2 was not changed in our study, it seems possible that the β3 subunit may be increased in diazepam-tolerant rats. However, the very low β3 subunit mRNA and protein contents in the FrPaM cortex of rats (36), as well as the results of preliminary experiments with specific PCR primers and internal standard for β3 subunit that failed to show significant differences in β3 subunit mRNA content in the FrPaM cortex of diazepam-tolerant rats, argue against this possibility.

Because studies with recombinant receptors and studies on γ2-suppressed transgenic mice indicate that BZ binding and BZ-induced positive allosteric modulation of GABA$_A$ receptor function is absent in GABA$_A$ receptors lacking the γ2 subunit protein, the persistence of a sufficient amount of γ2 subunit protein in the GABA$_A$ receptors of the FrPaM cortex of diazepam-tolerant rats is supported by the absence of change in the number and affinity of [^{3}H]flumazenil binding sites and by the normal anticonvulsant responsiveness of diazepam-tolerant rats to imidazenil.

It must be stressed, however, that just as the presence of a particular mRNA species does not confirm the presence of the corresponding subunit protein, the presence of a specific repertoire of subunit proteins in a given brain region or neuron does not necessarily indicate that these subunits are arranged with the same stoichiometry in the GABA$_A$ receptors. For example, it has been demonstrated that persistent stimulation of the GABA$_A$ receptors with GABA or BZs results in a shift of subunit polypeptides from the cell surface to an intracellular pool where the subunits lose their ability to be assembled in functional GABA$_A$ receptor complexes (55, 56).

Provisionally, our data can be interpreted as indicating that some changes in the subunit composition of the $GABA_A$ receptors may occur in selected neocortical areas of BZ-tolerant rats. It is impossible, however, to draw conclusions about the molecular nature of the events regulating these changes. This is due not only to the lack of correlation between mRNA and immunohistochemical data but also to stringent methodological considerations. In fact, because of the different affinities of the antibodies for their specific antigens, immunogold labeling allows either a comparison of the labeling density of a given subunit in a particular brain structure between various experimental groups of animals or of the immunolabeling density of a given subunit in different brain structures. Unfortunately, one cannot compare changes in the content of two different subunit proteins in the same brain area.

In conclusion, the results obtained with gold immunolabeling emphasize the need to increase our understanding of the relationship between changes in subunit mRNA content and changes in the expression of the respective protein content through the use of single-cell assay and double immunogold labeling with particles of different sizes in neurons isolated from neocortical areas of rats tolerant to BZ and rats not tolerant to a protracted imidazenil treatment.

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