Pergamon

PII: S0306-4522(99)00118-9

Neuroscience Vol. 93, No. 1, pp. 33–44, 1999

0306-4522/99 $20.00+0.00

BENZODIAZEPINE-MEDIATED REGULATION OF α1, α2, β1–3 AND γ2 $GABA_A$ RECEPTOR SUBUNIT PROTEINS IN THE RAT BRAIN HIPPOCAMPUS AND CORTEX*

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Abstract—Prolonged flurazepam exposure regulates the expression of selected (α1, β2, β3) $GABA_A$ receptor subunit messenger RNAs in specific regions of the hippocampus and cortex with a time-course consistent with benzodiazepine tolerance both *in vivo* and *in vitro*. In this report, the immunostaining density of six specific $GABA_A$ receptor subunit (α1, α2, β1–3 and γ2) antibodies was measured in the hippocampus and cortex, among other brain areas, in slide-mounted brain sections from flurazepam-treated and control rats using quantitative computer-assisted image analysis techniques. In parallel with the localized reduction in α1 and β3 subunit messenger RNA expression detected in a previous study, relative α1 and β3 subunit antibody immunostaining density was significantly decreased in flurazepam-treated rat hippocampal CA1, CA3 and dentate dendritic regions, and in specific cortical layers. Quantitative western blot analysis showed that β3 subunit protein levels in crude homogenates of the hippocampal dentate region from flurazepam-treated rats, an area which showed fairly uniform decreases in β3 subunit immunostaining (16–21%), were reduced to a similar degree (18%). The latter findings provide independent support that relative immunostaining density may provide an accurate estimate of protein levels. Consistent with the absence of the regulation of their respective messenger RNAs immediately after ending flurazepam administration, no changes in the density of α2, β1 or β2 subunit antibody immunostaining were found in any brain region. γ2 subunit antibody staining was changed only in the dentate molecular layer.

The selective changes in $GABA_A$ receptor subunit antibody immunostaining density in the hippocampus suggested that a change in the composition of $GABA_A$ receptors involving specific subunits (α1 and β3) may be one mechanism underlying benzodiazepine anticonvulsant tolerance. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: tolerance, flurazepam, quantitative immunohistochemistry, dentate gyrus, limbic system, CA1 pyramidal cells.

Although effective anxiolytics and hypnotics, the clinical use of benzodiazepines as anticonvulsants is limited by tolerance development during prolonged administration. A large body of evidence has indicated that benzodiazepine tolerance is related to a reduction in $GABA_A$ receptor-mediated fast inhibitory synaptic transmission (for reviews see Refs 2 and 27). The native $GABA_A$ receptor is a pentamer composed of combinations of homologous proteins derived from five subunit families with multiple variants (α1–6, β1–4, γ1–3, δ1 and ϵ1).[8,11,34] The $GABA_A$ receptor macromolecule contains binding domains for GABA and several allosteric modulators, including a well-characterized benzodiazepine binding site.[59] Benzodiazepines potentiate $GABA_A$ receptor-mediated inhibitory function by increasing the opening probability of the integral Cl^- channel,[52,62] and may increase Cl^- channel conductance.[13]

Although the synaptic mechanisms mediating benzodiazepine tolerance have not been determined, evidence has accumulated that a change in subunit-specific expression of neuronal $GABA_A$ receptor subtypes may modify inhibitory function during long-term benzodiazepine treatment.[21,22,27,29,30,44,49,65,66,80,81] Consistent with this hypothesis, time-dependent changes in the mRNA expression of specific $GABA_A$ receptor subunits (α1, β2 and β3) were localized to distinct hippocampal and cerebral cortical cell groups,[65,66] following one-week oral flurazepam administration. None the less, no direct link has been provided between changes in $GABA_A$ receptor subunit mRNA expression, changes in subunit protein expression and changes in $GABA_A$ receptor function.

The recent availability of subunit-specific antibodies for $GABA_A$ receptor subunit variants prompted these immunohistochemical studies. The specific goal was to establish whether there is a relationship between the reduced expression of specific $GABA_A$ receptor subunit mRNAs in hippocampal and cortical neurons,[65,66] and the expression of selected $GABA_A$ receptor subunit proteins (α1, α2, β1–3 and γ2). This relationship was investigated after an *in vivo* benzodiazepine treatment which reliably results in decreased $GABA_A$ receptor function in CA1 pyramidal cells,[73–78] but not dentate granule cells.[46] A reliable and sensitive computer-assisted image analysis method[23] was used to detect regional differences in $GABA_A$ receptor subunit protein immunostaining in the hippocampus, cortex and selected rat brain regions immediately after cessation of one-week oral flurazepam administration. The validity of this methodology to detect changes in rat brain protein levels was also assessed by western blot analysis of the β3 subunit antibody in crude homogenates of the dentate region of the hippocampus.

EXPERIMENTAL PROCEDURES

Animal model

Rats orally administered flurazepam in the drinking water for one week become tolerant to benzodiazepine suppression of pentylenetetrazole-induced seizures *in vivo*,[53,54] diazepam's action to potentiate

*Portions of this work have appeared in abstract form: *Soc. Neurosci. Abstr.* (1996) Abstract 22, 1285.

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Abbreviations: HRP, horseradish peroxidase; OD, optical density; TBS, Tris-buffered saline.

CA1 pyramidal cell population responses *in vitro*[74] and zolpidem's action to prolong miniature inhibitory postsynaptic current decay.[77] During oral flurazepam administration, rats become tolerant to benzodiazepine anticonvulsant actions, but not dependent. Rats do not show any overt behavioral effects or a spontaneous withdrawal syndrome, and only very mild precipitated abstinence signs, e.g., piloerection can be elicited with a very high dose (16 mg/kg, i.v.) of the antagonist, flumazenil.[68]

Adult, male Sprague–Dawley rats (Harlan, Indianapolis, IN; initial weight 180–225 g) received flurazepam for one week in 0.02% saccharin (100 mg/kg for three days, 150 mg/kg for four days) as their sole source of drinking water. Only rats that consumed a criterion dose,[66,75] i.e. a weekly average of >100 mg/kg/day, were used in these experiments. Control rats received saccharin water for the same length of time.[66,75] Rats were killed for brain section preparation immediately after ending one-week flurazepam administration, when maximal reductions in both α1 and β3 subunit mRNA expression were detected in the hippocampus and cortex.[24,65]

Brain section preparation

Sections (10 μm) cut from saline-perfused brains derived from flurazepam-treated and matched control rats were postfixed for detection of antibody staining. Immediately after the end of one-week flurazepam or saccharin treatment, rats were anesthetized with ketamine (80 mg/kg, i.m.) and transcardially perfused with 200 ml ice-cold 0.9% saline for 2 min at a constant rate (100 ml/min) to reduce the nonspecific immunolabeling prior to postfixation of sections. Rat brains were rapidly frozen in isopentane (−70°C) in an acetone/dry ice bath for 15 s. Parasagittal brain sections (10 μm) were cut on a cryostat at −12°C and thaw-mounted on to poly-L-lysine-coated slides for immunohistochemical studies.[27] Brain sections were stored at −70°C until used.

Polyclonal $GABA_A$ receptor subunit antibodies

Detailed descriptions of the polyclonal antibodies used were reported recently by Sperk *et al*.[61] The antibodies were raised in rabbits against rat specific amino acid residues of the α1, α2, β1–3 and γ2 subunits of the $GABA_A$ receptor (α1: 1–9; α2: 416–424; β1: 350–404; β2: 351–405; β3: 345–408; γ2: 316–352).[8,16] The α1 and α2 antibodies were coupled to keyhole limpet hemocyanine. The α1 antibody was directed against the N-terminus and contained a C-terminal cysteine. The α2 antibody was directed against the C-terminus and contained an N-terminal cysteine. Each antibody was affinity purified with corresponding peptides immobilized on thiopropyl-Sepharose 6B. The β1–3 and γ2 antibodies were against maltose binding protein fusion proteins purified using immobilized glutathione-*S*-transferase fusion proteins. The specificity of each $GABA_A$ receptor subunit antibody was reported previously.[16,41,61,70,79] The monoclonal anti-actin antibody used for western blot analysis, as a control for total protein, binds to a highly conserved region of actin, an abundant cytoskeletal protein.

Immunostaining for specific $GABA_A$ receptor subunit antibodies

For quantitative measurements in sections derived from experimental groups, tissue prefixation was undesirable, since the degree of fixation between treated and control rats would be difficult to control during perfusion. Therefore, flurazepam-treated and control sections were postfixed and handled in parallel throughout all procedures, as described previously.[24] Flurazepam-treated and control brain sections (10 μm) were warmed to room temperature under vacuum for 30 min. Brain sections were immersed in 4% paraformaldehyde (or plus 0.2% picric acid) for 8 min, then washed for 2×5 min in 300 mM Tris–HCl saline buffer (pH 7.2) at room temperature.[24,82] Fixed sections were blocked for 30 min at room temperature with 10% normal goat serum (v/v) plus 0.02% Triton X-100 (v/v) in Tris buffer prior to application of the primary antibody (α1: 3 μg/ml; α2, β1–3 and γ2: 10 μg/ml) and incubated overnight at 4°C. After three 5-min washes in buffer, matched flurazepam-treated and control sections were incubated with biotinylated anti-rabbit immunoglobulin G $F(ab')_2$ fragment (1:250, v/v, Boehringer Mannheim, Mannheim, Germany) for 1 h at 24°C. Sections were washed for 3×5 min in buffer and incubated with avidin–biotin–peroxidase complex (1:100 v/v; Vector Laboratories, Burlingame, CA) for 1 h. Immunostaining was visualized following immersion of matched flurazepam-treated and control sections in freshly made, filtered 0.06% diaminobenzidine (Sigma Chemical Co., St Louis, MO). Hydrogen peroxide (0.02%) was added immediately before the chromogen reaction was initiated. The chromogen reaction was allowed to proceed for no more than 5 min and was identical for flurazepam-treated and control groups. Intense staining was avoided, as overreaction would interfere with quantitation.[27] Sections were dehydrated through 75%, 95% and 100% ethanol (4 min each), cleared with xylene and coverslipped with Permount (Fisher, Pittsburgh, PA) for acquisition of digitized images. The primary antibody was left out of the incubation in adjacent sections as a negative control. Additional negative controls were used for the β subunit antibodies, i.e. sections were incubated in the presence of each β subunit primary antibody preadsorbed with 10 μg/ml of the respective fusion proteins for 24 h.

Quantitative measurement of $GABA_A$ receptor subunit antibody staining

Immunostaining intensities in the brain regions of interest were measured using a computer-assisted image analysis system.[24] A light box (Northern Light, Imaging Research, St Catherines, Ontario, Canada) provided constant illumination underneath the slide. Light intensity was adjusted to provide optimal contrast among different brain subregions on tissue sections, but was held constant across groups. The images of the hippocampus and other brain areas of interest were acquired with a high-resolution CCD camera (Sierra Scientific, Sunnyvale, CA) with the aid of NIH Image software (v. 1.59). Images of flurazepam-treated (n=8) and matched control (n=8) brain sections were digitized at the same time, with the same degree of illumination under identical conditions. Grey values, which reflect immunostaining density, were measured over the regions of interest on the digitized images.[24] Specific immunostaining over an area of interest was defined by subtraction of the background grey level value measured over the white matter area nearest the region of interest. Background values were not significantly different between groups (P>0.05). All data are expressed as mean±S.E.M. of the raw grey level values. Regional variations in the distribution of mean $GABA_A$ receptor subunit protein staining were compared using a repeated measures ANOVA. Post hoc comparisons were made with orthogonal contrasts.

Quantitative western blot analysis of dentate β3 subunit protein levels

Immunoblot analysis using the β3 subunit antibody was carried out in the dentate region of the hippocampus microdissected from separate groups of one-week flurazepam-treated (n=6) and control (n=6) rats, killed immediately after drug or vehicle removal. Rats were decapitated and the left hippocampus was dissected into ice-cold pre-gassed (95% O_2/5% CO_2) buffer containing (in mM): NaCl 120, KCl 5.0, $MgSO_4$ 1.3, NaH_2PO_4 1.2 $CaCl_2$ 2.4, $NaHCO_3$ 26, D-glucose 10 (pH 7.3). Six to eight 500–750 μm transverse sections were cut on a Vibratome from the entire hippocampus and stored in ice-cold buffer. The dentate gyrus (~8 mg tissue, wet weight), including the polymorph region, was dissected under a microscope (×20, A.O. Instr., Buffalo, NY) on ice and homogenized 20 strokes in 100 μl of 0.32 M sucrose in a Potter–Elvejheim homogenizer. Crude homogenates were stored at −80°C until used. Protein concentrations were determined using bovine serum albumin as standard.[33]

Protein from each flurazepam-treated and control rat dentate gyrus was applied to alternate lanes of each of two 11% sodium dodecyl sulfate–polyacrylamide gels. One gel was used for subsequent detection of the β3 subunit protein (~10 μg/lane). The second gel was used for subsequent detection of actin (~5.25 μg/lane; Boehringer Mannheim Corp., Indianapolis, IN), to control for total protein, using identical dilutions of the crude dentate homogenates. The molecular weights of protein samples were estimated from pre-stained standards (14,300–200,000; Gibco BRL, Gaithersurg, MD). The protein was transferred from the gels to Immobilon (polyvinylidene fluoride) membranes (Fisher Biotech, Pittsburgh, PA) for 1 h (90 mA) using semi-dry transfer techniques. Membranes were blocked overnight at 4°C in 2.5% non-fat dry milk in Tris-buffered saline (TBS)/0.1% Tween-20 (Fisher Biotech). Membranes were rinsed for 2×5 min in blocking solution and incubated for 1 h with the primary antibody (β3 subunit: 1 μg/ml; actin: 1 μg/ml) in blocking solution containing 0.02% sodium azide at room temperature with shaking. Membranes were rinsed for 3×5 min with TBS/Tween-20. In preliminary immunoblot analyses, anti-β3 subunit and anti-actin immunoreactivity on

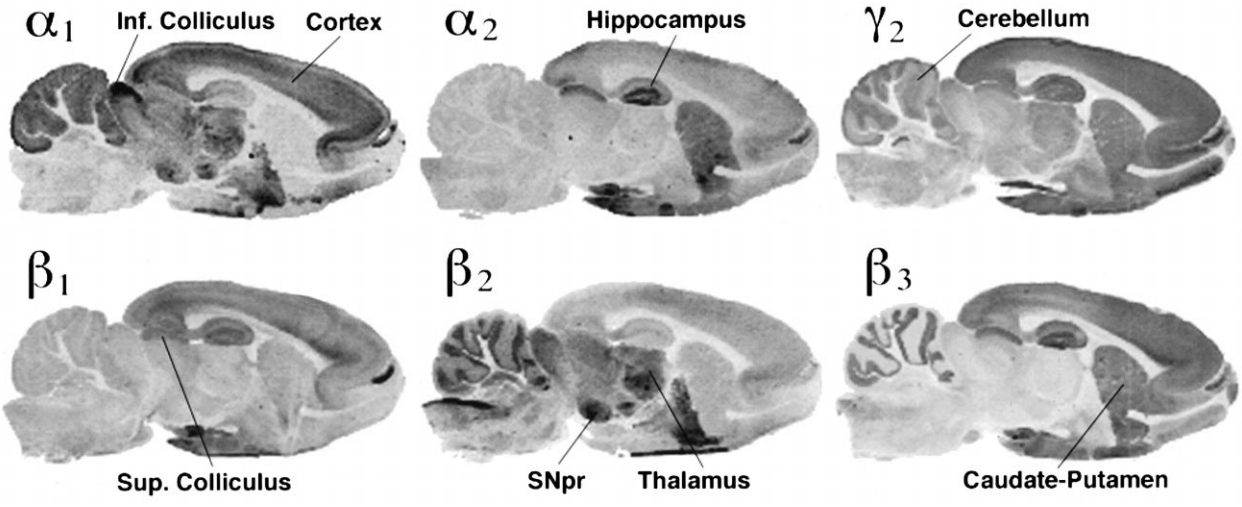

Fig. 1. Digital images of serial parasagittal brain sections from a representative control rat. Sections (10 μm) were immunostained with one of six specific $GABA_A$ receptor subunit subunit antibodies (α1, α2, β1–3, γ2). Regional variations in immunohistochemical staining were examined using high-resolution computer-assisted image analysis. The times for the chromogen reactions were non-saturating and not always comparable among subunits. Furthermore, since differences exist among antibody affinities and since the staining protocol was carried out independently for each antibody, only relative comparisons of staining density can be made within sections. Immunostaining density was compared in subregions of the hippocampus and cortex, as well as the caudate–putamen, thalamus, substantia nigra pars reticulata (SNpr), inferior and superior colliculus, and cerebellum. The α1 and β2 subunit antibodies had a similar staining distribution in the midbrain and had a higher staining density in the cortex than the hippocampus. The α1 and β2 subunit antibodies were lower in the hippocampus than for other immunopositive regions. The α2 and β3 antibodies had a similar immunostaining distribution in parasagittal sections except that the β3 antibody labeled the granule cell layer in the cerebellum, whereas α2 subunit antibody staining was almost absent in the cerebellum. α2, β1, β3 and γ2 subunit antibodies all strongly labeled the hippocampus with varying distributions. α2, β3 and γ2 subunit antibodies also strongly labeled the caudate–putamen and superior colliculus, whereas α1, β2 and γ2 antibodies labeled the thalamus, inferior colliculus and substantia nigra pars reticulata, where γ2 subunit staining was relatively low. All subunit antibodies labeled cortical layers with varying densities. The cerebellar granule cell layer was strongly immunopositive for β2 and β3 subunit antibodies, while the cerebellar molecular layer was strongly positive for α1 and γ2 antibodies. The α2 and β1 subunit antibodies showed little or no staining in either cerebellar layer.

membranes was detected using a secondary antibody conjugated to alkaline phosphatase. For quantification of β3 subunit and actin immunoreactivity on membranes, the enhanced chemiluminescence method (ECL, Amersham, Arlington Heights, IL) was used. Membranes were incubated with the appropriate horseradish peroxidase (HRP)-linked secondary antibody [β3 subunit: anti-rabbit immunoglobulin G–HRP (New England Biolabs, Beverly, MA), 1:2000 in TBS/1% gelatin/0.02% sodium azide; actin: anti-mouse immunoglobulin G–HRP (New England Biolabs), 1:5000 in TBS/gelatin/sodium azide] for 1 h at room temperature. After three 5-min washes in Tris/Tween-20, membranes were reacted with the chemiluminescent substrate (Pierce, Rockford, IL) for 1 min. Reacted membranes were exposed to RX X-ray film (Fuji Medical Systems, U.S.A., Stamford, CT) for 5 s to 20 min and developed.

The density of the bands reflecting the β3 subunit protein or actin protein was quantified using a densitometer (Model GS-670, BioRad, Hercules, CA) and Molecular Analyst Software (v. 1.1, BioRad). Film density, measured as transmittance, was expressed as volume-adjusted optical density (OD/mm^2). Adjusted OD values, reflecting β3 subunit protein levels, were determined for each dentate gyrus. β3 subunit protein level estimates were normalized to the respective individual density values reflecting actin protein levels and were expressed as a ratio. The difference between the means of the ratios calculated from densitometric measures of protein levels derived from control ($n=6$) and flurazepam-treated ($n=6$) dentate gyrus was analysed using a one-tailed paired Student's *t*-test with a significance level of $P \leq 0.05$.

RESULTS

Relative subunit antibody immunostaining patterns

Relative α1, α2 and γ2 subunit antibody immunostaining patterns in the hippocampus and other brain regions were similar to those extensively described previously using both the light microscopic and electron microscopic techniques (Fig. 1),[15,18,19,42,60,61,82] and therefore will not be described in further detail. The distribution of the β2 subunit has been described previously by Moreno *et al.*,[40] using an antibody to an alternative epitope. The comparative distributions of the β1–3 antibodies are provided in detail in Sperk *et al.*[61]

Benzodiazepine regulation of $GABA_A$ receptor subunit protein immunostaining

Computer-assisted image analysis techniques, developed previously in our laboratory,[24] were used to evaluate the intensity of immunostaining in 10-μm parasagittal rat brain sections (Table 1). Immunostaining density over the hippocampus and other selected brain areas was compared in sections derived from one-week flurazepam-treated and control rats to determine the relative amount of each antigen present ($n=6$–8/group). Grey values over the areas of interest on digitized images of parasagittal brain sections were used to represent the density of α1, α2, β1–3 and γ2 $GABA_A$ receptor subunit antibody immunostaining and to statistically compare staining between experimental groups (Tables 1–3). For comparisons of changes in levels of protein expression among the six subunit antibody staining patterns, the data in the text and in Figs 4 and 7 are expressed as a percentage of control expression.

Regulation of α subunit antibody immunostaining

In comparison to other brain areas, e.g., the cerebral cortex, cerebellum, thalamus and inferior colliculus, α1 antibody immunostaining density in parasagittal sections was lower in the hippocampal formation (Fig. 1). A decrease in α1 subunit antibody immunostaining density was found in several subregions of the hippocampus (Table 1, Figs 2, 4) and cortex (Table 2, Figs 3, 6) in sections derived from flurazepam-treated rats versus their matched controls. In the hippocampus,

Table 1. Subunit antibody immunostaining in the hippocampus

Subregion		α1	α2	β1	β2	β3	γ2
CA1							
SO	Control	12.8±0.8	17.6±1.2	27.1±2.0	5.0±0.3	31.9±2.1	53.0±1.3
	FZP-treated	10.1±0.7	17.8±1.5	29.4±2.3	5.1±0.4	25.9±2.2	51.2±1.5
	P value	0.01*	0.94	0.42	0.88	0.04*	0.35
SP	Control	10.9±0.7	16.9±0.9	25.6±2.7	5.1±0.3	28.1±1.2	40.2±1.4
	FZP-treated	9.5±0.5	18.3±1.8	25.6±3.3	4.8±0.4	23.2±3.2	39.7±1.6
	P value	0.09	0.50	0.99	0.62	0.12	0.81
SR	Control	9.9±0.6	14.9±1.2	24.4±2.1	4.7±0.2	28.8±1.8	58.1±1.7
	FZP-treated	7.9±0.5	17.0±1.3	25.3±2.2	5.0±0.4	21.5±2.7	55.2±1.9
	P value	0.02*	0.24	0.75	0.45	0.03*	0.24
SL-M	Control	20.1±2.9	24.8±1.9	35.3±1.6	7.0±0.3	35.8±2.2	48.9±1.9
	FZP-treated	13.0±0.7	27.3±2.4	35.7±2.0	6.8±0.4	30.8±2.2	46.4±1.1
	P value	0.01*	0.40	0.85	0.64	0.10	0.24
CA2							
SO	Control	11.3±1.8	16.9±1.1	32.9±2.7	4.7±0.4	22.0±1.5	50.7±0.9
	FZP-treated	8.0±0.5	17.7±1.3	30.7±3.3	4.4±0.3	18.1±1.9	49.7±1.7
	P value	0.06	0.63	0.74	0.54	0.11	0.58
SP	Control	11.4±2.0	18.0±1.2	30.5±2.3	5.0±0.4	22.8±1.4	40.8±1.5
	FZP-treated	8.3±0.7	19.6±1.9	25.0±3.6	4.6±0.2	18.6±2.0	42.3±1.3
	P value	0.10	0.45	0.38	0.35	0.08	0.42
SR	Control	8.1±2.0	16.2±1.3	31.1±2.5	4.4±0.4	22.7±1.8	56.0±1.1
	FZP-treated	6.3±0.5	18.6±1.3	28.1±3.0	4.4±0.4	16.9±1.8	54.9±1.6
	P value	0.30	0.19	0.64	1.00	0.03*	0.56
CA3							
SO	Control	10.8±2.1	23.8±1.8	32.8±1.9	4.9±0.4	32.2±1.0	56.7±1.0
	FZP-treated	6.7±0.4	24.8±1.8	33.6±2.0	5.1±0.3	29.7±0.8	54.4±1.3
	P value	0.03*	0.68	0.77	0.64	0.06	0.17
SP	Control	5.3±0.8	18.0±1.5	21.1±2.0	3.9±0.3	19.9±0.9	43.4±1.3
	FZP-treated	3.8±0.4	21.0±2.6	22.5±2.3	3.8±0.3	19.4±2.4	41.4±1.3
	P value	0.06	0.32	0.62	0.84	0.82	0.26
SL	Control	10.3±1.6	26.0±1.9	33.4±2.0	4.8±0.3	37.4±1.9	61.1±1.2
	FZP-treated	6.4±0.5	28.7±1.8	34.1±1.9	4.9±0.2	32.1±1.5	58.4±1.2
	P value	0.01*	0.29	0.80	0.97	0.03*	0.12
Dentate							
PC (CA4)	Control	5.2±0.7	24.4±2.4	23.6±2.1	3.9±0.4	30.8±1.5	42.9±1.0
	FZP-treated	4.0±0.4	29.9±2.9	25.7±2.8	3.7±0.3	24.4±2.6	41.5±1.1
	P value	0.10	0.14	0.51	0.73	0.04*	0.33
GC	Control	8.4±0.9	23.4±2.0	26.7±2.7	4.2±0.4	33.2±1.3	33.2±1.5
	FZP-treated	6.6±0.6	28.3±2.3	28.1±2.9	3.9±0.5	27.3±2.6	31.4±1.4
	P value	0.10	0.11	0.71	0.68	0.05*	0.36
OM	Control	16.3±1.4	—	—	—	47.7±2.1	—
	FZP-treated	10.7±0.6	—	—	—	40.1±1.9	—
	P value	<0.01*				0.01*	
IM (IM+OM)	Control	13.1±1.3	(35.1±2.6)	(36.0±1.4)	(5.0±0.3)	43.7±2.3	(55.7±2.0)
	FZP-treated	8.8±0.6	(38.9±2.6)	(37.1±2.1)	(5.0±0.3)	35.4±2.1	(51.3±1.1)
	P value	<0.01*	0.31	0.62	0.95	0.01*	0.05*

Mean(±S.E.M.) relative grey level.
*Significant difference between control and flurazepam (FZP)-treated groups, $P \leq 0.05$.
Control: α1, $n=6$; α2, β1, β3, γ2, $n=7$; β2, $n=8$; flurazepam-treated: all antibodies, $n=8$.
CA1–CA3: SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SL-M, stratum lacunosum-moleculare; SL, stratum lucidum.
Dentate: PC, polymorph (CA4) cells; GC, granule cells; OM, outer molecular layer; IM, inner molecular layer.

the mean relative grey value was significantly decreased in flurazepam-treated brain sections in CA1 dendritic regions, i.e. a 21% decrease in stratum oriens, a 21% decrease in stratum radiatum and a 35% decrease in stratum lacunosum (Table 1, Fig. 4). No significant differences were found between groups in the pyramidal cell layer (Table 1). A significant decrease in staining density was also found in the CA3 strata oriens (−38%) and lucidum (−38%), but not the CA3 stratum pyramidale (Table 1, Fig. 4). In the dentate gyrus, a comparison of flurazepam-treated and control rats indicated a significant decrease in the mean grey value between groups in the inner (−33%) and outer (−35%) molecular layers, but not in the granule cell or polymorph cell layers (Fig. 4). Preliminary experiments in 20-μm parasagittal sections derived from a second group of treated and control rats ($n=8$/group) revealed similar significant decrease in α1 subunit antibody immunostaining in hippocampal subregions (data not shown).

A significant decrease in α1 subunit antibody immunostaining density was also detected in the cortical layers of parasagittal sections. The pattern of change was most consistent in layers V and VI across all cortical regions measured, i.e. the frontal, parietal and occipital cortices (Figs 3, 6). There was also a significant decrease in α1 immunostaining in parietal cortex layer IV (−26%) in flurazepam-treated sections (Fig. 6, Table 2). A similar non-significant trend

Table 2. Subunit antibody immunostaining in the cerebral cortex

Layer		α1			β3		
		Frontal	Parietal	Occipital	Frontal	Parietal	Occipital
I	Control	30.6±3.9	28.0±2.5	31.5±2.2	35.8±3.1	35.2±2.1	35.8±2.1
	FZP-treated	23.4±1.8	22.9±2.8	24.1±3.7	27.1±3.9	28.4±3.4	29.3±3.8
	P value	0.07	0.18	0.11	0.09	0.09	0.14
II/III	Control	25.3±2.7	23.3±2.9	18.6±2.1	36.2±2.5	35.3±1.3	35.9±2.0
	FZP-treated	19.2±1.5	19.4±2.6	16.9±2.3	27.8±3.3	27.6±3.3	29.7±3.0
	P value	0.04*	0.30	0.58	0.05*	0.04*	0.09
IV	Control	33.1±3.1	37.9±4.3	31.3±2.8	37.9±2.3	36.6±1.5	37.0±2.3
	FZP-treated	28.4±2.1	28.1±2.6	24.9±2.5	30.0±3.4	29.3±2.7	30.5±2.8
	P value	0.18	0.04*	0.07	0.30	0.03*	0.08
V	Control	27.8±2.1	28.8±3.1	28.9±3.3	31.9±1.1	28.6±2.3	27.6±2.5
	FZP-treated	23.3±1.1	22.5±2.1	22.4±1.5	18.6±2.4	25.5±2.5	24.4±2.6
	P value	0.04*	0.09	0.05*	<0.01*	0.35	0.36
VI	Control	25.0±1.0	25.9±3.0	25.8±2.1	34.6±1.1	29.5±2.2	31.0±2.0
	FZP-treated	20.6±0.9	19.2±1.4	19.7±0.9	21.4±2.7	27.9±2.4	28.5±2.2
	P value	<0.01*	0.03*	<0.01*	<0.01*	0.61	0.38

Mean (±S.E.M.) relative grey level.
*Significant difference between control and flurazepam (FZP)-treated, $P \leq 0.05$.
Control: α1, $n=6$; β3, $n=7$; flurazepam-treated: α1, $n=8$; β3, $n=8$.

Table 3. Subunit antibody immunostaining in other brain regions

Brain region		α1	α2	β1	β2	β3	γ2
Caudate–putamen	Control	—	19.4±1.7	—	—	23.0±1.0	48.8±0.8
	FZP-treated	—	19.4±1.4	—	—	21.7±0.6	48.2±1.0
	P value		0.99			0.21	0.61
Thalamus	Control	21.0±3.6	—	—	11.3±0.4	—	47.7±0.8
	FZP-treated	18.4±2.0	—	—	11.8±0.7	—	47.2±0.8
	P value	0.48			0.56		0.70
SNpr	Control	15.0±1.7	—	—	15.4±1.4	—	27.6±3.0
	FZP-treated	13.0±0.7	—	—	17.1±0.8	—	25.6±1.9
	P value	0.23			0.27		0.55
Inferior colliculus	Control	29.6±2.8	—	—	19.2±1.2	—	41.7±2.5
	FZP-treated	29.1±1.9	—	—	18.1±1.7	—	38.7±1.9
	P value	0.88			0.57		0.32
Superior colliculus							
SGS	Control	—	14.6±1.6	40.2±2.6	—	26.3±1.5	47.6±3.4
	FZP-treated	—	14.7±1.0	39.5±1.6	—	26.3±1.3	45.0±1.5
	P value		0.98	0.81		0.99	0.44
SO	Control	—	—	—	15.7±1.6	—	—
	FZP-treated	—	—	—	14.6±1.6	—	—
	P value				0.61		
SGI	Control	15.2±1.1	—	—	13.8±1.2	—	34.5±3.1
	FZP-treated	14.9±0.7	—	—	14.2±1.1	—	32.3±1.5
	P value	0.78			0.80		0.48
Cerebellum							
GC	Control	27.6±2.0	—	—	19.8±0.8	33.5±2.1	34.4±1.1
	FZP-treated	24.9±1.4	—	—	19.7±1.0	33.0±2.3	35.5±1.1
	P value	0.23			0.92	0.87	0.46
ML	Control	26.5±1.9	—	—	—	—	48.7±1.4
	FZP-treated	24.3±1.7	—	—	—	—	47.9±1.7
	P value	0.37					0.73

Mean (±S.E.M.) relative grey level.
*Significant difference between control and flurazepam (FZP)-treated groups, $P \leq 0.05$.
Control: α1, $n=6$; α2, β1, β3, γ2, $n=7$; β2, $n=8$; flurazepam-treated: all antibodies, $n=8$.
SNpr, substantia nigra pars reticulata.
Superior colliculus: SGS, stratum griseum superficiale; SO, stratum opticum; SGI, stratum griseum intermediate.
Cerebellum: GC, granule cell layer; ML, molecular layer.

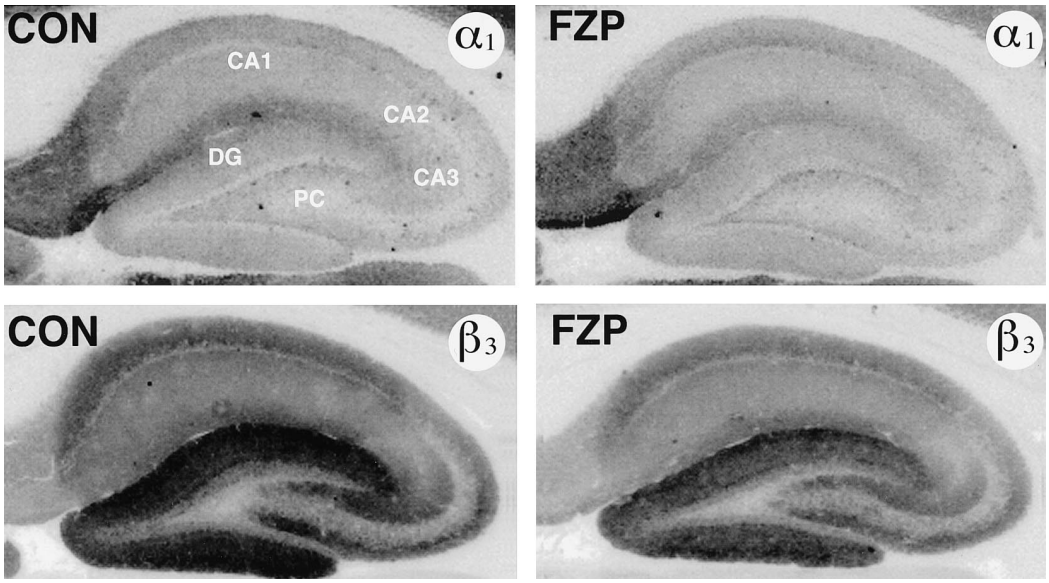

Fig. 2. Relative distribution of α1 and β3 subunit antibody immunostaining in representative 10-μm parasagittal sections of hippocampus derived from control (CON) and one-week flurazepam-treated (FZP) rats. There were significant reductions in immunostaining density in strata oriens (α1 and β3), radiatum (α1 and β3) and lacunosum-moleculare (α1) of the CA1 pyramidal cell region, and strata oriens (α1) and lucidum of the CA3 pyramidal cell region. Immunostaining was also decreased in the dentate molecular layer (α1 and β3), and in the granule cell (β3) and polymorph cell (PC; β3) regions (Table 1).

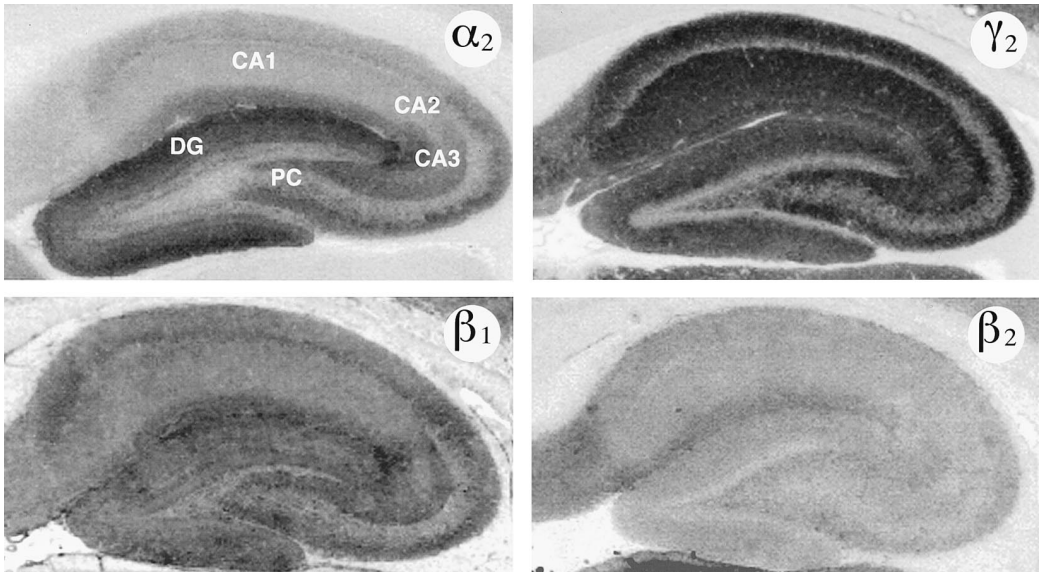

Fig. 3. Relative distributions of α2, γ2, β1 and β2 subunit antibody immunostaining in representative 10-μm parasagittal sections of the hippocampus derived from control rats are presented for comparison with the distributions of α1 and β3 antibody staining in Fig. 2. Only the γ2 subunit antibody showed a small, but significant, reduction in immunostaining density in the molecular layer of the dentate gyrus (DG). There were no other significant reductions in α2, β1 or β2 subunit antibody immunostaining density in any other pyramidal cell (CA1–CA3) or dentate gyrus subregion (Table 1).

was seen in the occipital cortex, whereas frontal cortex layers II/III showed a significant reduction (−24%) in α1 subunit immunostaining (Table 2). No significant differences in α1 subunit immunostaining density were detected in the cerebellar molecular or granule cell layers. There were also no between-group differences in α1 subunit antibody immunostaining density in the other brain regions measured, i.e. the thalamus or cerebellum (Table 3).

The cerebral cortex, caudate–putamen, superior colliculus and dentate regions were among those brain regions most densely labeled with the α2 subunit antibody (Fig. 1). The immunostaining density of the α2 subunit antibody was measured in the hippocampus (Table 1, Fig. 4) and caudate–putamen, the two strongly immunoreactive brain areas, as well as in the cerebral cortex and cerebellum. There were no significant differences in immunostaining between groups in any of the brain regions measured (Tables 2, 3, Fig. 6).

Regulation of β subunit antibody immunostaining

The β1 subunit antibody stained the cerebral cortex, superior colliculus, midbrain and hippocampus most intensely (Fig. 1). There were no differences in β1 immunostaining between

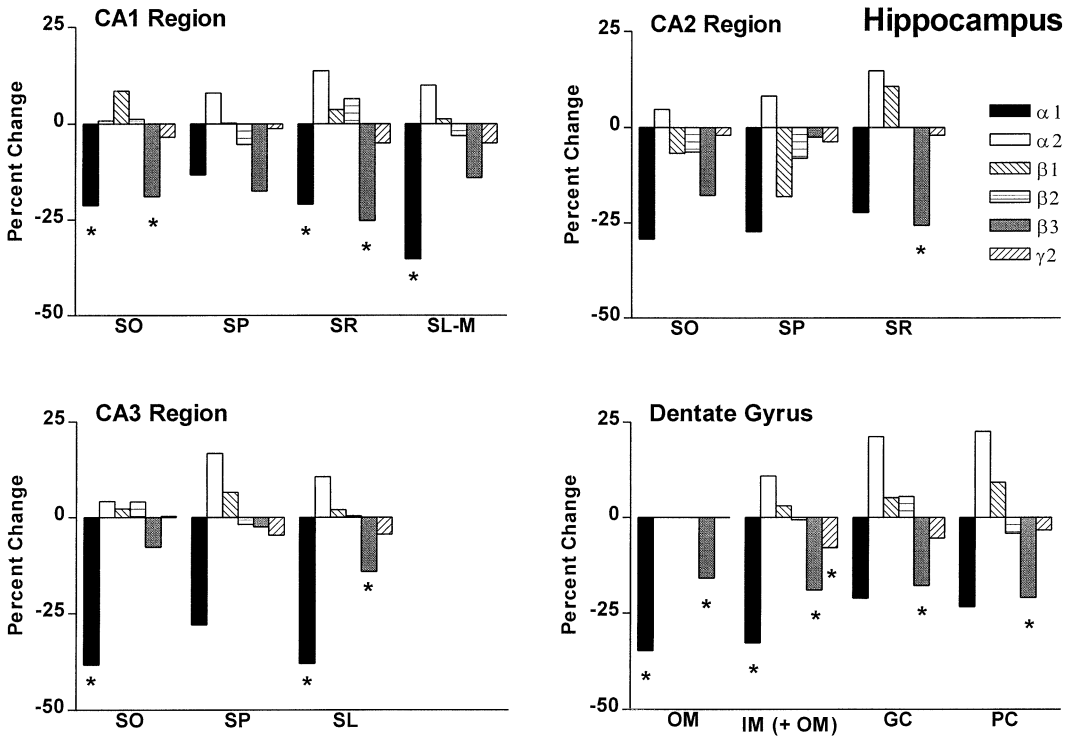

Fig. 4. The percentage change in the distribution of $GABA_A$ receptor subunit antibody immunostaining in the hippocampus following one-week oral flurazepam administration. Relative grey level was measured over digitized images of immunostained hippocampal subregions [CA1 and CA2: SO, stratum oriens; SP, stratum pyramidale; SR; stratum radiatum; SL-M, stratum lacunosum-moleculare; CA3: SO, stratum oriens; SP, stratum pyramidale; SL, stratum lucidum; dentate gyrus: OM, outer molecular layer; IM, inner molecular layer; GC, granule cell layer; PC, polymorph cell layer (formerly CA4 pyramidal cell layer)] from control or one-week flurazepam-treated rats (Table 1). Bars: black, α1; white, α2; left cross-hatch, β1; horizontal line, β2; grey, β3; right cross-hatch, γ2. Asterisks denote significant differences between control and flurazepam-treated mean (± S.E.M.) relative grey level values, $P \leq 0.05$.

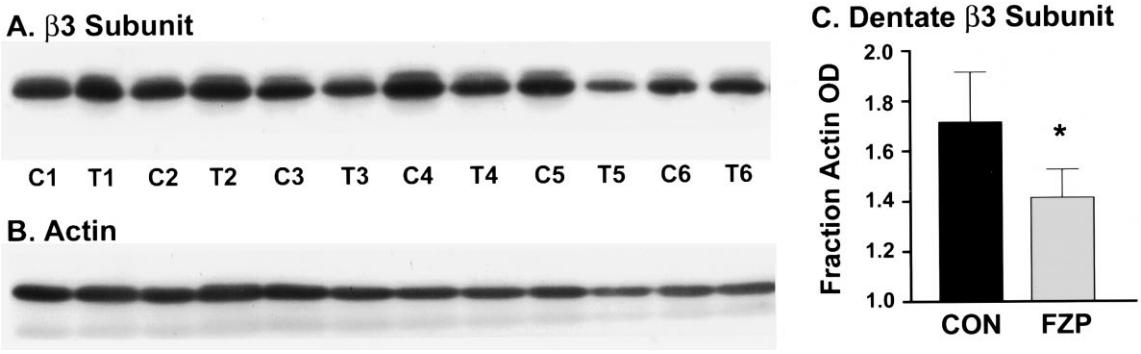

Fig. 5. Crude homogenates of the dentate gyrus microdissected from the hippocampus of flurazepam-treated ($n=6$) and control ($n=6$) rats were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and western blot analysis. (A) Immunoblot using a specific polyclonal antibody to the $GABA_A$ receptor β3 subunit. The signals over dentate samples (10 μg protein/lane) from control (C1–C6) and flurazepam-treated (T1–T6) rats were visualized on film using the enhanced chemiluminescence technique. A broad band, representing the N-glycosylated β3 subunit, centered at about 56,000 mol. wt, was revealed after 20 min exposure. (B) Immunoblot of a specific anti-actin antibody to the identical crude dentate homogenates (5.25 μg protein/lane), as a control for total protein. The film was exposed for 5 s, revealing a band of mol. wt 42,000. Owing to the strong chemiluminescence, the fainter bands in B were the result of errant re-exposure during removal of the film from the cassette and were not used for analysis. The OD/mm^2 measurements derived from the β3 subunit immunoblot were normalized to those from the actin immunoblot and expressed as a ratio. (C) β3 subunit protein level, as a fraction of actin protein per dentate gyrus sample, was compared between control and flurazepam-treated rats. The signal was quantified by measuring the volume-adjusted optical density (OD/mm^2) of each band from a densitometric image of film transmittance. The asterisk represents a significant ($P=0.047$) 18% reduction in the mean (±S.E.M.) β3 subunit protein level in the dentate gyrus after one-week flurazepam treatment.

groups in any hippocampal region (Table 1, Fig. 6), the cerebral cortex (Table 2, Fig. 7) or cerebellum (Table 3).

The whole-brain regional distribution pattern of β2 subunit immunostaining was similar to the α1 subunit antibody, although somewhat lower in intensity in the hippocampus and cortex (Fig. 1). As with the β1 subunit antibody, there were no significant differences in β2 subunit antibody immunostaining between groups in the hippocampus (Table 1), cerebral cortex (Table 2), caudate–putamen, thalamus or cerebellum (Table 3).

The β3 subunit antibody prominently immunostained many brain regions, including the cerebral cortex, midbrain, caudate–putamen, superior colliculus, the cerebellar granule cell layer and the hippocampus (Fig. 1). In contrast to

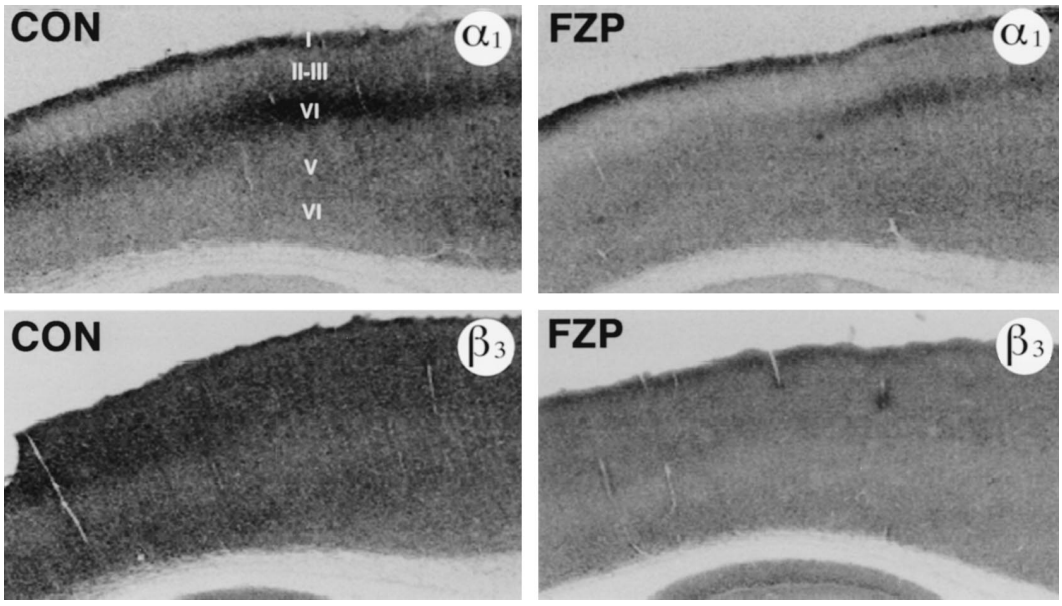

Fig. 6. Relative distributions of α1 and β3 subunit antibody immunostaining in representative 10-μm parasagittal sections of the parieto-occipital cortex derived from control (CON) and one-week flurazepam-treated (FZP) rats. Relative grey level was measured over digitized images of each cortical layer: I, II/III, IV, V and VI. There were significant reductions in both α1 and β3 subunit immunostaining density in several layers of the frontal, parietal and occipital cortices (Table 2).

immunostaining with the other β subunits, β3 subunit immunoreactivity was significantly decreased in the CA1 region of flurazepam-treated rat hippocampus. A significant decrease in staining density was measured in strata oriens (−19%) and radiatum (−25%), but not strata pyramidale (−17%) and lacunosum (−14%) (Table 1, Fig. 4). In the CA3 region, a significant decrease in immunostaining density was also found in stratum lucidum (−14%) (Table 1, Fig. 4). In the dentate gyrus, β3 antibody staining density was significantly decreased in flurazepam-treated brain sections in the inner (−19%) and outer (−16%) molecular layers, as well as the granule cell (−18%) and polymorph cell (−21%) layers (Table 1, Fig. 4).

In comparison to α1 subunit immunostaining, the β3 subunit showed a similar pattern of significantly decreased immunostaining in the frontal cortex layers, although the magnitude of the decrease was two-fold greater in layers V and VI (II/III, −23%; V, −42%; VI, −38%; Table 2, Fig. 7). Similarly, the pattern of decrease in β3 immunostaining was like that of the α1 antibody, i.e. significantly decreased in layer IV of the parietal cortex (−19%) with a similar trend in layer IV of the occipital cortex (Table 2, Fig. 7). However, unlike the α1 subunit antibody, the β3 subunit antibody staining was not modified in layers V or VI of the parietal and occipital cortices following chronic flurazepam treatment (Table 2, Fig. 7). There were also no changes in β3 subunit antibody staining in the caudate–putamen or cerebellar granule cell layer (Table 3, Fig. 1).

Western blot analysis of β3 subunit protein level in the dentate gyrus

A broad band centered at mol. wt 56,000, representing the N-glycosylated $GABA_A$ receptor β3 subunit,[6,57] was detected on immunoblots using both the secondary antibody conjugated to alkaline phosphatase and that linked to HRP (Fig. 5A). A minor band, at mol. wt ~53,000, was visualized when the alkaline phosphatase colorometric reaction was extended (data not shown). The anti-actin antibody recognized a single band (mol. wt 42,000) using the enhanced chemiluminescence method (Fig. 5B). A comparison of the densitometric readings from the film overlying dentate homogenate samples derived from flurazepam-treated (n=6) and control rats (n=6) is shown in Fig. 5C. There was a significant (P=0.047) 18% reduction in β3 subunit protein levels in the dentate gyrus immediately after ending flurazepam treatment. The degree of reduction in β3 subunit protein levels in dentate homogenates was similar to that detected using immunohistochemical methods in brain sections (Fig. 4; −16% to −21%).

Regulation of γ2 subunit antibody immunostaining

γ2 subunit antibody staining was widely distributed among brain regions in parasagittal sections (Fig. 1), with equivalent immunostaining densities in the CA1–CA3 and dentate regions (Fig. 3). γ2 subunit antibody staining was not significantly changed between groups in any pyramidal cell region in the hippocampus (Table 1, Fig. 4). In the dentate gyrus molecular layer, the γ2 staining density in flurazepam-treated brain sections showed a small (−8%), but significant, decrease in density across the molecular layer in comparison to control sections (Table 1, Fig. 4). No significant differences between groups were detected in the dentate granule cell layer (Table 1). The density of γ2 subunit immunoreactivity was also not significantly decreased between groups in any layer of the cerebral cortex (Table 2, Fig. 7), caudate–putamen, thalamus or cerebellum (Table 3).

DISCUSSION

Using specific $GABA_A$ receptor polyclonal antibodies, these experiments were designed to investigate the regulation of selected subunit proteins by benzodiazepine allosteric

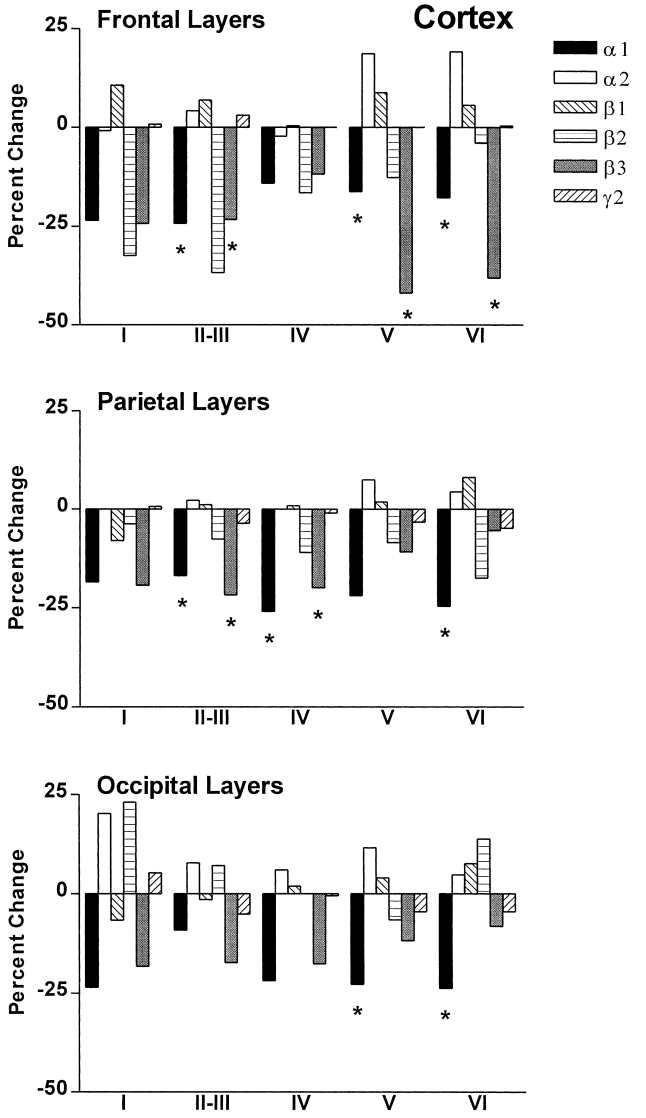

Fig. 7. The percentage change in the distribution of $GABA_A$ receptor subunit antibody immunostaining in parietal cortex layers following one-week flurazepam administration. Relative grey level was measured over digitized images of cortical layers I, II/III, IV, V and VI from control or one-week flurazepam-treated rats (Table 2). Bars: black, α1; white, α2; left cross-hatch, β1; horizontal line, β2; grey, β3; right cross-hatch, γ2. Asterisks denote significant differences between control and flurazepam-treated mean (±S.E.M.) relative grey level values, $P \leq 0.05$.

modulators. The gross brain regional distributions of subunit antibody staining in this report were consistent with the patterns of associated subunit antibodies identified by immunoprecipitation and immunofluorescence studies.[14,35,39] In the parasagittal plane sampled (Fig. 1), α1, β2 and γ2 subunit proteins were enriched in the thalamus, pallidum, inferior colliculus and in certain cortical areas. The α2, β3 and γ2 subunits appeared more prevalent in the dentate gyrus, caudate–putamen, superior colliculus and outer layers of cortex. These findings conform to previous reports of enriched mRNA expression in these same brain regions.[71] The somatic versus dendritic co-localization of certain subunit proteins in the hippocampus and cortex is probably related to the localized assembly of a variety of $GABA_A$ receptor subtypes and the functional heterogeneity of the GABA and allosteric modulator responses in hippocampal[1,30,36,45,58,67] and cortical neurons.[25,56] The discrete localization of α1, α2, β1–3 and γ2 subunit proteins reinforced the likelihood that different $GABA_A$ receptor subtypes exist on different subcellular domains of hippocampal and cortical principle cell types. For example, the α2 subunit protein, previously localized to axo-axonic synapses on the axon initial segment of the hippocampal pyramidal cell,[43] may be more likely to be associated with the β1 or β3 subunit.[4,39] The α1 subunit, found on a subset of synapses on pyramidal cell somata and on proximal and distal dendrites,[4,43] has frequently been associated with the β2 and γ2 subunits,[5,14] and was prominently localized to hippocampal interneurons.[17,34,38,39] The regulation of α1 and β3 subunit proteins in pyramidal cell dendritic regions suggests that an α1β3γ2-containing $GABA_A$ receptor subtype may be regulated following long-term flurazepam treatment *in vivo*.

The discrete suppression of $GABA_A$ receptor function in CA1 pyramidal cells during prolonged flurazepam administration[47,73,75–78] and the associated regionally localized, time-dependent reductions in the expression of specific $GABA_A$ receptor subunit mRNAs[65,66] warranted quantitative immunohistochemical studies of selected hippocampal and cortical $GABA_A$ receptor subunit proteins (α1, α2, β1–3 and γ2). Taken together with the findings of *in situ* hybridization studies,[65,66] the results of these quantitative immunohistochemical studies suggested that changes in the expression of specific $GABA_A$ receptor mRNAs (α1 and β3 subunits) were mirrored by changes in their respective subunit proteins. The reliability and validity of using image analysis to make quantitative measurements of changes in chromogen staining density in brain sections were established previously.[24] None the less, whether such changes in $GABA_A$ receptor subunit antibody immunostaining density reflect changes in subunit protein levels could not be assessed directly in tissue sections. Accordingly, it was necessary to further validate this methodology using an independent method, quantitative western blot analysis, to detect changes in subunit protein levels. The dentate gyrus was selected for analysis for two reasons. First, among all hippocampal subregions, the dentate gyrus can be most reliably microdissected. More importantly, the dentate showed the most consistent, significant reductions in β3 subunit antibody staining across the molecular layers (inner: 19%; outer: 16%) and the granule cell (18%) and polymorph cell regions (−21%) (Fig. 4), and thus was expected to be least sensitive to small localized variations in β3 subunit protein levels. The finding that β3 subunit protein levels were significantly reduced in crude homogenates of the dentate region of the hippocampus using immunoblot analyses (Fig. 7) further established the validity of computer-assisted image analysis of immunohistochemical staining to measure relative subunit protein levels. The similarity in degree of reduction (~18%) in subunit protein using both methods (Figs 4, 5) suggested that immunostaining density may provide an accurate estimate of subunit protein levels in various brain regions. Although there are limitations as to the cellular resolution which can be achieved, the simple image analysis techniques described provided a rapid, reliable screen of regional changes in protein levels,[24] without the specialized equipment required for more quantitative approaches at the subcellular level.[42,60]

This is the first report of the differential, localized regulation of $GABA_A$ receptor proteins (α1, α2, β1–3 and γ2) in the hippocampus after chronic benzodiazepine

treatment paralleling changes in their respective mRNAs.[64,67] The relationship between changes in receptor protein and alterations in $GABA_A$ receptor properties must be resolved. Both [^{3}H]flunitrazepam[9,69] and [^{3}H]zolpidem (unpublished observations) binding was reduced in the CA1 region of the hippocampus immediately after flurazepam treatment was stopped. GABA/benzodiazepine receptors were also functionally uncoupled.[9] Changes in the number or affinity of binding sites for GABA and benzodiazepine agonists have been demonstrated inconsistently after various benzodiazepine treatments.[2,5,9,26,50,68] Alternatively, a reduced allosteric interaction, i.e. decreased functional coupling, between GABA and benzodiazepine binding sites on native[9,17,23,51,64] and recombinant[32,50,72] $GABA_A$ receptors is a uniform finding. Studies of GABA/benzodiazepine receptor coupling in aged rats suggested that a decreased allosteric modulation with age was related to the decreased amount of α1 subunit protein.[55] Conversely, although GABA agonist exposure resulted in $GABA_A$ receptor internalization,[8] neither receptor down-regulation or internalization,[50] nor changes in α1 subunit mRNA or protein expression,[32] could account for benzodiazepine agonist-mediated uncoupling in recombinant expression systems. Structural or other post-translational modifications of the receptor, i.e. phosphorylation of specific $GABA_A$ subunits,[34] were proposed mechanisms responsible for both benzodiazepine receptor down-regulation[5] and GABA/benzodiazepine receptor uncoupling,[50] although reports conflict.[31]

The role of a switch in $GABA_A$ receptor subunits in mediating functional changes in GABA receptor function remains speculative and will require an analysis of the time-course of changes in α1 and β3 (and β2) subunit proteins in the hippocampus. Such a switch could result in a change in the pharmacological response to GABA or to benzodiazepine receptor ligands. A decreased potency of the α1 subunit-selective imidazopyridine, zolpidem, to potentiate GABA currents in dissociated CA1 pyramidal cells of chronic diazepam-treated rats,[28] and the reduced ability of zolpidem to potentiate the decay of CA1 pyramidal cell miniature inhibitory postsynaptic currents,[77] suggest that the function of α1-containing $GABA_A$ receptor subtypes may be selectively impaired after prolonged flurazepam treatment. The finding that α1 subunit staining was reduced in CA1 dendritic regions is consistent with this possibility. Whether a reduction in the β3 subunit protein, as reflected in the reduction of β3 antibody staining, may play a permissive or a more integral role in the reduction of $GABA_A$ receptor function remains to be determined. The β subunit of the $GABA_A$ receptor plays a critical role in subcellular targeting of assembled receptors and may have important functions to regulate receptor localization or clustering.[10,46] Hadingham *et al.*[20] reported little influence of β subunit substitution on GABA-mediated currents in two heterologous expression systems, whereas transient transfection of β3 versus β1 or β2 subunits into HEK-293 cells increased GABA affinity 2.5-fold in α1β*x*γ2 recombinant receptors.[12] Thus, the increase in β2 subunit mRNA expression in CA1 pyramidal cells two days after ending flurazepam administration, concomitant with the decreased β3 subunit mRNA[24] and β3 subunit protein expression in CA1 dendritic regions, could account for the 2.5-fold decreased $GABA_A$ agonist potency in the CA1 region of the hippocampus of flurazepam-tolerant rats,[74] although a similar β subunit switch might be expected to increase, rather than decrease, zolpidem affinity.[4]

CONCLUSIONS

Although the time-courses of localized reductions in α1 and β3 subunit proteins were consistent with changes in their respective subunit mRNAs, the dynamic relationship among the regulation of receptor subunit mRNAs, subunit protein and the final insertion of functional receptors at the synapse is a current topic of inquiry. Studies of the effects of GABA exposure on chick cortical neuron $GABA_A$ receptor α1 subunit mRNA and α1 polypeptide revealed a delayed, use-dependent decline in α1 subunit translation,[38] consistent with the decreased α1 subunit mRNA.[3] Whether a similar delay in the repression of $GABA_A$ receptor subunit biosynthesis may occur following benzodiazepine exposure requires further investigation.[63] Such a mechanism could explain the one-week delay in the reduction of α1 and γ2 mRNA in the cortex of mice administered lorazepam for two weeks,[29] in contrast to the reduction in benzodiazepine receptor number and GABA-mediated chloride uptake following only one week of benzodiazepine treatment.[37] It will be important to determine the temporal pattern of changes in subunit protein expression following chronic benzodiazepine administration in comparison to the time-course of the change in the expression of $GABA_A$ receptor subunit mRNAs.[17,22,27,29,44,65,66,80,81] Such information may help to clarify the relationship between changes in protein expression (e.g., of α1, β2 and β3 subunits) and changes in the GABAergic function of some (CA1 pyramidal cells), but not other (CA3 pyramidal cells and dentate granule cells), hippocampal principal cell types in benzodiazepine-tolerant rats.[28,47,48,75,76,78]

Acknowledgements—We would like to thank Eugene Orlowski, Scott Lilly and Bradley Van Sickle for technical assistance, and Dr Linda Dokas for expert assistance with immunoblot analyses and helpful comments regarding the final manuscript. This work was supported by National Institutes of Health grants R01DA04075 and K02DA00180 to E.I.T.

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(*Accepted* 24 *February* 1999)