Pergamon

Neuroscience Vol. 91, No. 1, pp. 327–341, 1999

PII: S0306-4522(98)00516-8
0306-4522/99 $20.00+0.00

TEMPORAL AND REGIONAL REGULATION OF α1, β2 AND β3, BUT NOT α2, α4, α5, α6, β1 OR γ2 $GABA_A$ RECEPTOR SUBUNIT MESSENGER RNAS FOLLOWING ONE-WEEK ORAL FLURAZEPAM ADMINISTRATION

E. I. TIETZ,* X. HUANG, S. CHEN and W. F. FERENCAK III

Department of Pharmacology, Medical College of Ohio, 3035 Arlington Avenue, Toledo, OH 43614-5804, U.S.A.

Abstract—The effect of prolonged benzodiazepine administration on $GABA_A$ receptor subunit (α1–6, β1–3, γ2) messenger RNAs was investigated in the rat hippocampus and cortex, among other brain areas. Rats were orally administered flurazepam for one week, a protocol which results in benzodiazepine anticonvulsant tolerance *in vivo*, and in the hippocampus *in vitro*, in the absence of behavioral signs of withdrawal. Autoradiographs of brain sections, hybridized with [^{35}S]oligoprobes *in situ*, were examined immediately (day 0) or two days after drug treatment, when rats were tolerant, or seven days after treatment, when tolerance had reversed, and were compared to sections from pair-handled, vehicle-treated controls. α1 subunit messenger RNA level was significantly decreased in CA1 pyramidal cells and dentate granule cells at day 0, an effect which persisted only in CA1 neurons. Decreased "α1-specific" silver grain density over a subclass of interneurons at the pyramidal cell border suggested concomitant regulation of interneuron $GABA_A$ receptors. A reduction in β3 subunit messenger RNA levels was more widespread among hippocampal cell groups (CA1, CA2, CA3 and dentate gyrus), immediately and two days after treatment, and was also detected in the frontal and parieto-occipital cortices. Changes in β2 subunit messenger RNA levels in CA1, CA3 and dentate gyrus cells two days after ending flurazepam treatment suggested a concomitant up-regulation of β2 messenger RNA. There was a trend toward an increased level of α5, β3 and γ2 subunit messenger RNAs in CA1, CA3 and dentate gyrus cells, which was significant for the β3 and γ2 subunit messenger RNAs in the frontal cortex seven days after ending flurazepam treatment. There were no flurazepam treatment-induced changes in any other $GABA_A$ receptor subunit messenger RNAs.

The messenger RNA levels of three (α1, β2 and β3) of nine $GABA_A$ receptor subunits were discretely regulated as a function of time after ending one-week flurazepam treatment related to the presence of anticonvulsant tolerance, but not dependence. The findings suggested that a localized switch in the subunit composition of $GABA_A$ receptor subtypes involving these specific subunits may represent a minimal requirement for the changes in $GABA_A$ receptor-mediated function recorded previously at hippocampal CA1 GABAergic synapses, associated with benzodiazepine anticonvulsant tolerance. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: benzodiazepines, GABA, hippocampus, dependence, tolerance, *in situ* hybridization.

Several classes of clinically effective anticonvulsants exert their actions at different allosteric modulatory sites on the $GABA_A$ receptor (cf. Ref. 62). Continual use of the benzodiazepine class leads to a reduction in clinical effectiveness, i.e. tolerance. It is well established that benzodiazepine tolerance is associated with a dysfunction of GABAergic neurotransmission (for reviews see Refs 3, 21 and 30). $GABA_A$ receptors gate fast inhibitory synaptic transmission via an integral chloride ion channel. The native receptor is a pentamer with unknown stoichiometry, though it is probably composed of 2α, 2β and a γ (or δ) subunit.[2,9,39] Multiple variants of the five $GABA_A$ receptor subunit families have been identified (α1–6, β1–4, γ1–4, δ1, ϵ1), which may confer different Cl^- channel properties and distinct GABA and benzodiazepine pharmacological properties.[13,14,39,57,63,70,71]

$GABA_A$ receptors have a dense, heterogeneous distribution throughout the CNS, particularly in the cortex and hippocampus.[48] At least five patterns of $GABA_A$ receptor subunit association have been identified *in vivo*[5,17,18,41,44,52,75] and certain subunit combinations have been associated with specific neuronal populations.[23,41,44] The heterogeneity and subcellular compartmentalization of $GABA_A$ receptor subunits suggest that multiple, functionally distinct $GABA_A$ receptor subtypes may be assembled on individual neurons.[44]

A change in expression of the genes encoding $GABA_A$ receptor subunits, and thus a switch in the subunit composition of $GABA_A$ receptor subtypes, has been proposed to contribute to the dysfunction at

*To whom correspondence should be addressed.

Abbreviations: DG, dentate gyrus; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SSC, standard saline citrate.

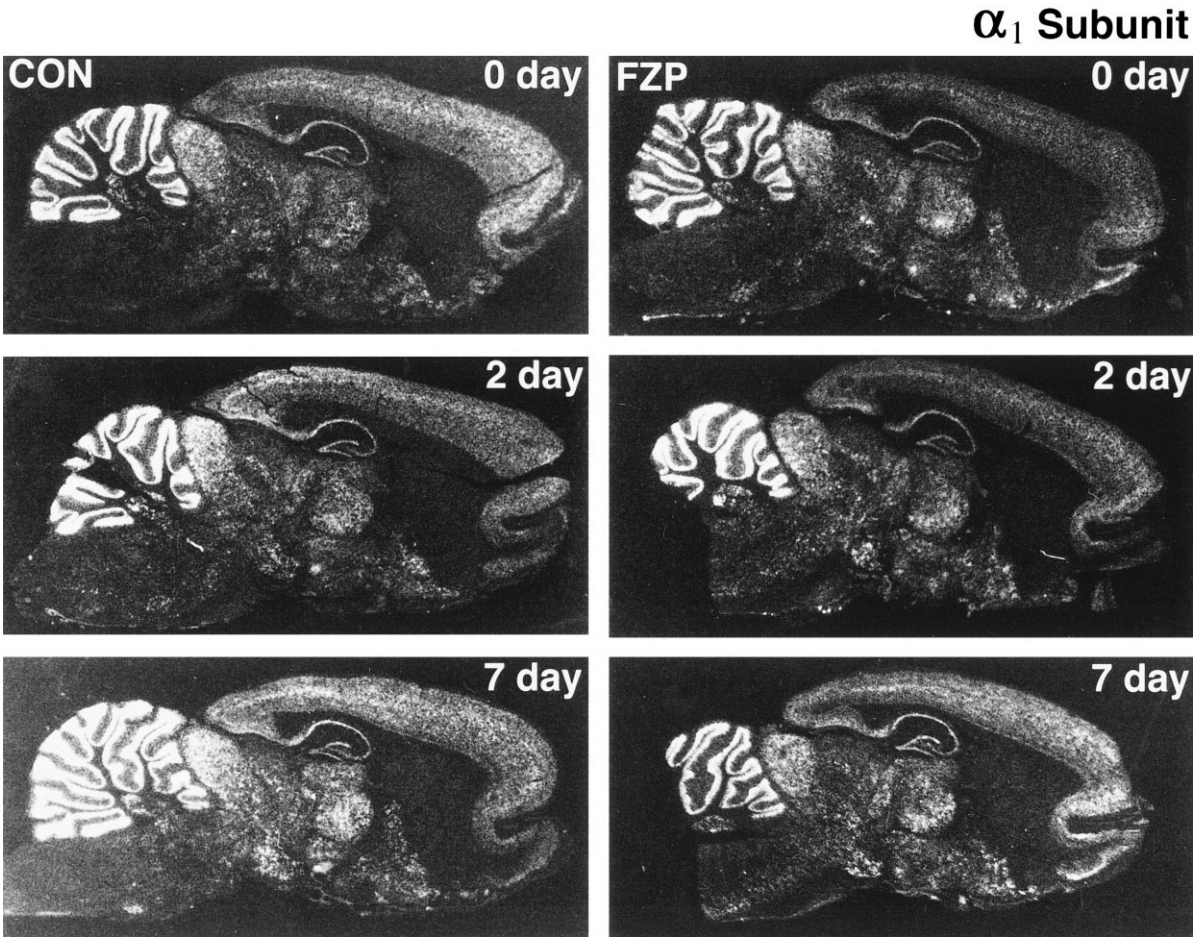

Fig. 1. Representative dark-field photomicrographs of autoradiographs of sagittal brain sections hybridized with an antisense [^{35}S]oligoprobe for the α1 subtype mRNA of the $GABA_A$ receptor. Sections (10 μm) were cut from one-week flurazepam-treated (FZP) rats killed immediately (day 0), two or seven days after ending oral drug administration and compared to sections from matched control rats (CON). Immediately after the end of treatment, there was a significant decrease in the relative gray level on autoradiographs localized over CA1 pyramidal cells and dentate granule cells. This difference only persisted in CA1 pyramidal cells two days after treatment and was no longer present seven days after ending treatment, when rats are no longer tolerant *in vivo* (Table 1).

GABAergic synapses associated with benzodiazepine tolerance. Variable expression of receptor subunits could alter GABA receptor function and GABA or benzodiazepine pharmacology, and could be manifested behaviorally as a decreased CNS sensitivity to the drug following repeated or prolonged administration. Studies aimed at establishing such a role for changes in $GABA_A$ receptor subunit composition associated with benzodiazepine tolerance have yielded conflicting findings.[20,31,33,42,73] One potential difficulty may be that some benzodiazepine treatments produce not only tolerance, but also physical dependence. It could then become difficult to differentiate molecular mechanisms underlying reduced neuronal responsiveness to benzodiazepines, i.e. functional tolerance, from similar or additional changes in $GABA_A$ receptors occurring in response to repeated or continued receptor activation, but associated with physical dependence. Dependence may be manifested as behavioral hyperexcitability following drug withdrawal or precipitated, i.e. by antagonist administration. Another difficulty in interpreting the results of a variety of previous studies is the identification of a common anatomical locus or loci mediating functional tolerance. Moreover, whereas studies of tolerance mechanisms in heterologous expression systems following prolonged agonist exposure may yield important insights into the possible relationship between biochemical and molecular mechanisms underlying pharmacological or functional changes at $GABA_A$ receptors, these mechanisms cannot be directly ascribed to tolerance *in vivo*.

One-week oral flurazepam treatment produces tolerance to benzodiazepine actions to suppress pentylenetetrazole-induced seizures *in vivo*,[59,60] but not dependence.[67] Tolerance was also demonstrated *in vitro* in a model system established in the hippocampus of flurazepam-treated rats, by a reduced

sensitivity of GABA-mediated responses in CA1 pyramidal cells to benzodiazepine receptor agonists.[78,81,83] $GABA_A$ receptor agonist potency was also reduced in CA1 pyramidal cells.[78] Moreover, among the principal hippocampal cell types studied following chronic benzodiazepine treatments with a range of electrophysiological approaches, GABAergic function was shown to be selectively impaired in CA1 pyramidal cells.[53,54,79–82] Furthermore, the temporal pattern of the changes in GABA function detected in CA1 pyramidal cells following one-week flurazepam treatment[53,81,82,84] paralleled the appearance and disappearance of anticonvulsant tolerance *in vivo*.[59,60] Using *in situ* hybridization methods, we had previously demonstrated localized decreases in α1, but not α5 and γ2, mRNA levels in the cortex and CA1 region of the hippocampus two days after ending flurazepam administration. In order to establish whether the expression of specific $GABA_A$ receptor subunits may be discretely regulated with a similar time-course as the temporal pattern of tolerance detected *in vivo* and *in vitro*, the relative expression of nine $GABA_A$ receptor subunit (α1, α2, α4, α5, α6, β1, β2, β3 and γ2) mRNAs was investigated in the hippocampus, among other brain areas.

EXPERIMENTAL PROCEDURES

Chronic benzodiazepine treatment

After a two-day adaptation period, male Sprague–Dawley rats (180–200 g initial weight; Harlan, Indianapolis, IN) were offered flurazepam hydrochloride in 0.02% saccharin water for one week (100 mg/kg for three days, 150 mg/kg for four days). Rats treated using this protocol are tolerant,[59,60] but show no signs of spontaneous or precipitated withdrawal.[67] Though rats are offered what are apparently relatively "high" doses of flurazepam, benzodiazepine levels in the brain achieved during one-week oral flurazepam administration, expressed in diazepam equivalents (161.1 ± 35.9 ng diazepam/g brain; 0.57 μM),[53,66,77,78] are similar to or less than those achieved with other chronic benzodiazepine treatments (200–275 ng diazepam/g brain; 0.7–1.0 μM).[20,42,58] At the end of the drug administration period, rats were killed for brain section preparation. Rats were killed immediately (day 0) after ending flurazepam administration or offered saccharin water for two or seven days until they were killed. Control rats received the saccharin solution for the same lengths of time. Changes in mRNA levels were anticipated to be at or near maximal immediately after drug removal, or still decreased two days after drug removal, when impaired $GABA_A$ receptor inhibition was detected in the hippocampus in the absence of residual benzodiazepine metabolites.[77] Seven days after the end of treatment, when tolerance has reversed, the electrophysiological correlates of tolerance have also disappeared.[53,77,81,82,84]

In accordance with the Public Health Service Policy on Human Care and Use of Laboratory Animals and the Animal Welfare Act, all efforts were made to minimize animal suffering, to reduce the numbers of animals used and to utilize alternatives to *in vivo* techniques.

In situ *hybridization histochemistry*

Oligonucleotide probes. Oligoprobes (45-mers) complimentary to rat cDNA amino acid residues (α1, 342–356;[34] α2, 341–357;[35] α3, 361–375;[40] α4, 15–30 of the signal peptide;[74] α5, 355–369,[40] α4 according to Khrestchatisky *et al.*;[34] α6, 342–356;[38] β1, 382–396;[79] β2, 382–396;[79] β3, 380–394;[79] γ2, 338–352[61]) were synthesized by Oligos Etc. (Willsonville, OR) according to Wisden *et al.*[75] Probes were 3′ end-labeled with [^{35}S]dATP (12.5 mCi/ml; E. I. Dupont, Boston, MA) with terminal deoxytransferase (Boehringer Mannheim, Indianapolis, IN). ^{35}S-Labeled oligoprobe was separated from unincorporated [^{35}S]dATP by centrifugation on a Bio-Spin chromatography column (BioRad, Richmond, CA). Labeling efficiency ranged from 67% to 90% for all oligoprobes, except the α3 probe (~ 50%). Coupled with the low levels of the α3 subunit mRNA, particularly in the hippocampus,[72,75] preliminary experiments indicated that the very weak α3 antisense [^{35}S]oligoprobe hybridization signal on autoradiographs could not be reliably quantified. Therefore, between-group comparisons with the α3 probe were not carried out.

Tissue preparation. Brains were dissected and rapidly frozen in isopentane in an acetone–dry ice bath. Sagittal serial sections (10 μm) were cut through the hippocampal formation, 2.4–2.9 mm lateral to the midline, according to Paxinos and Watson.[50] Sections were thaw-mounted on to poly-L-lysine-coated slides and stored at −70°C until processed for *in situ* hybridization histochemistry. Prior to hybridization, sections were rapidly brought to room temperature under vacuum and fixed for 5 min in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). For each oligoprobe, parallel serial sections were selected from flurazepam-treated and control groups based on their lateral level of section.[50] Sections were rinsed 3 × 5 min in sodium phosphate buffer. Sections were immersed for 10 min in 0.1 M triethanolamine containing 0.25% acetic anhydride then rinsed for 10 min in 2 × standard saline citrate (SSC; 1 × SSC = 0.15 M NaCl, 0.15 M sodium citrate, pH 7.0), dehydrated through an ethanol series (70%, 80%, 95%), defatted in chloroform and stored under 95% ethanol at −20°C until used. For each [^{35}S]oligoprobe, one section per rat from both experimental and control groups were batch processed in parallel and handled identically across all time-points.

Hybridization. Sections were air-dried and prehybridized with 80 μl/slide of hybridization buffer [50% (v/v) formamide, 4 × SSC, 1 × Denhardt's reagent, 500 mg/μl heat denatured Herring sperm DNA, 250 ml yeast tRNA and 10% dextran sulfate containing 10 mM dithiothreitol] under parafilm in a humidified (2 × SSC) chamber. Slides treated with RNase for 30 min at 37°C prior to prehybridization were used as negative controls.[66] Sections were hybridized at 42°C in a humidified chamber with 50 ml/slide hybridization buffer containing 10 mM dithiothreitol and 1 × 10^6 d.p.m. ^{35}S-labeled antisense oligoprobe/section under glass coverslips with parafilm bridges. Additional slides were incubated with sense α1 and β3 [^{35}S]oligoprobes. Coverslips were removed in 2 × SSC. Sections were washed to a final stringency of 0.5 × SSC at 52–60°C according to the calculated oligoprobe T_m. Sections were dehydrated for 5 min in 70% and 95% ethanol containing 300 mM ammonium acetate (pH 5.5), followed by 100% ethanol. Slides were dried in a vacuum desiccator and apposed to Biomax MR film (Eastman Kodak Co., Rochester, NY). The development of the image on the film was tracked with sections hybridized with increasing ratios of oligoprobe labeled with [^{35}S]dATP to "cold" dATP (40%, 80% and 100%) to help insure that the signal was adequate, but not saturated. Coronal sections, hybridized with the ^{35}S-labeled α1 antisense probe, were dipped in NTB-2 emulsion (Eastman Kodak Co., Rochester, NY) as described previously[66] for grain counting over presumptive interneurons in the hippocampal CA1 region. Autoradiographic film and emulsion were

Table 1. Time-dependent α1, β2 and β3 subunit messenger RNA levels in flurazepam-treated rat brain

		α1 subunit			β2 subunit			β3 subunit		
		0 days	2 days	7 days	0 days	2 days	7 days	0 days	2 days	7 days
Groups	Control	$n = 8$	$n = 7$	$n = 8$	$n = 8$	$n = 8$	$n = 7$	$n = 8$	$n = 7$	$n = 8$
	FZP treated	$n = 8$	$n = 8$	$n = 8$	$n = 8$	$n = 7$	$n = 8$	$n = 8$	$n = 7$	$n = 8$
Hippocampus proper										
CA1 pyramidal cells	Control	79.1 ± 2.7	67.0 ± 3.3	71.6 ± 5.1	40.1 ± 5.0	33.4 ± 3.4	42.9 ± 6.3	81.9 ± 6.1	98.7 ± 4.3	80.4 ± 3.7
	FZP treated	67.7 ± 4.3	59.6 ± 2.4	67.4 ± 4.3	39.4 ± 7.8	43.8 ± 3.7	45.6 ± 6.9	62.3 ± 7.1	82.3 ± 6.4	89.0 ± 5.0
	P value	0.03*	0.04*	0.53	0.93	0.05*	0.76	0.04*	0.04*	0.16
CA2 pyramidal cells	Control	70.9 ± 6.7	61.3 ± 4.3	64.1 ± 4.4	30.3 ± 4.6	27.2 ± 3.2	31.6 ± 3.8	72.3 ± 6.8	92.0 ± 5.5	77.8 ± 2.5
	FZP treated	69.7 ± 5.6	61.1 ± 1.4	61.6 ± 3.5	30.3 ± 6.9	26.9 ± 3.1	39.0 ± 5.2	61.6 ± 4.6	75.8 ± 3.9	80.5 ± 3.4
	P value	0.89	0.87	0.65	1.0	0.93	0.25	0.04*	0.02**	0.51
CA3 pyramidal cells	Control	53.7 ± 3.0	46.4 ± 2.1	46.7 ± 3.5	27.9 ± 2.7	28.4 ± 3.8	24.6 ± 4.0	86.5 ± 5.9	97.7 ± 4.0	82.7 ± 4.2
	FZP treated	46.2 ± 3.3	45.6 ± 2.3	48.1 ± 2.3	25.4 ± 6.4	39.0 ± 3.2	30.8 ± 4.3	70.6 ± 4.3	84.3 ± 2.9	97.4 ± 3.6
	P value	0.10	0.66	0.73	0.69	0.04*	0.29	0.04*	0.04*	0.01*
Dentate gyrus										
Polymorph cells (CA4 pyramidal cells)	Control	45.5 ± 3.8	30.9 ± 0.9	38.9 ± 3.3	24.3 ± 3.0	22.5 ± 3.0	24.3 ± 4.0	93.6 ± 6.7	98.7 ± 4.2	88.2 ± 5.2
	FZP treated	37.5 ± 3.4	32.0 ± 2.1	39.2 ± 2.2	25.2 ± 6.4	25.9 ± 2.8	28.9 ± 4.0	74.0 ± 6.3	81.7 ± 3.9	92.6 ± 4.7
	P value	0.11	0.37	0.94	0.89	0.40	0.40	0.11	0.001*	0.51
Granule cells	Control	72.2 ± 3.2	55.3 ± 3.1	65.1 ± 4.1	32.5 ± 3.4	29.4 ± 2.9	34.0 ± 4.5	113.9 ± 7.2	127.5 ± 3.1	105.7 ± 5.6
	FZP treated	58.1 ± 4.1	51.0 ± 3.3	63.7 ± 4.5	32.7 ± 7.0	37.9 ± 2.6	34.9 ± 4.7	89.7 ± 8.1	108.9 ± 3.7	113.6 ± 3.8
	P value	0.01*	0.26	0.82	0 97	0.04*	0.88	0.03*	0.001*	0.23
Cortex										
Frontal	Control	53.7 ± 5.9	47.5 ± 5.0	54.8 ± 5.0	30.9 ± 3.6	23.0 ± 3.3	24.8 ± 9.0	35.1 ± 4.3	50.5 ± 3.6	24.2 ± 9.0
	FZP treated	44.6 ± 7.2	46.6 ± 3.0	45.6 ± 5.5	22.3 ± 6.1	23.5 ± 2.8	22.4 ± 2.6	22.0 ± 2.6	49.5 ± 2.4	34.0 ± 3.1
	P value	0.31	0.48	0.20	0.20	0.91	0.46	0.01*	0.80	0.02*
Parieto-occipital	Control	47.7 ± 3.1	42.8 ± 4.0	47.1 ± 2.3	25.1 ± 2.2	22.9 ± 2.6	27.4 ± 4.3	31.1 ± 3.1	48.6 ± 4.0	25.7 ± 3.0
	FZP treated	41.9 ± 1.6	33.6 ± 3.0	41.1 ± 3.3	23.0 ± 3.6	16.4 ± 2.2	20.3 ± 3.5	24.0 ± 3.6	41.9 ± 2.0	25.0 ± 2.7
	P value	0.09	0.24	0.13	0.60	0.06	0.38	0.14	0.14	0.86
Caudate–putamen	Control	—	—	—	—	—	—	29.7 ± 3.8	49.1 ± 2.7	26.4 ± 3.2
	FZP treated	—	—	—	—	—	—	21.0 ± 1.7	45.1 ± 0.6	30.8 ± 2.4
	P value	—	—	—	—	—	—	0.03*	0.15	0.27
Thalamus	Control	53.8 ± 2.6	44.2 ± 3.6	51.8 ± 3.5	45.4 ± 6.6	50.3 ± 4.2	59.2 ± 6.4	—	—	—
	FZP treated	51.7 ± 2.9	45.3 ± 3.1	57.1 ± 3.3	44.0 ± 6.3	44.2 ± 2.6	58.3 ± 6.5	—	—	—
	P value	0.57	0.63	0.26	0.87	0.21	0.78	—	—	—
Cerebellum										
Granule cells	Control	140.8 ± 4.5	121.1 ± 4.7	133.4 ± 6.4	90.4 ± 119	69.4 ± 12.8	86.7 ± 11.0	74.2 ± 5.9	92.6 ± 7.7	75.2 ± 3.6
	FZP treated	132.5 ± 3.0	115.5 ± 7.7	124.1 ± 3.9	96.8 ± 14.4	61.1 ± 5.0	105.6 ± 12.2	79.7 ± 8.7	74.2 ± 4.9	72.0 ± 8.1
	P value	0.12	0.52	0.21	0.72	0.52	0.38	0.60	0.05*	0.70

Mean (± S.E.M.) relative gray level. FZP, flurazepam; *n*, number of rats.
*Significant difference between control and flurazepam-treated groups, $P \leq 0.05$.
Note: image acquisition conditions were identical within, and not between, temporal groups; therefore, comparisons of relative gray level should be made between experimental groups, not between temporal groups.

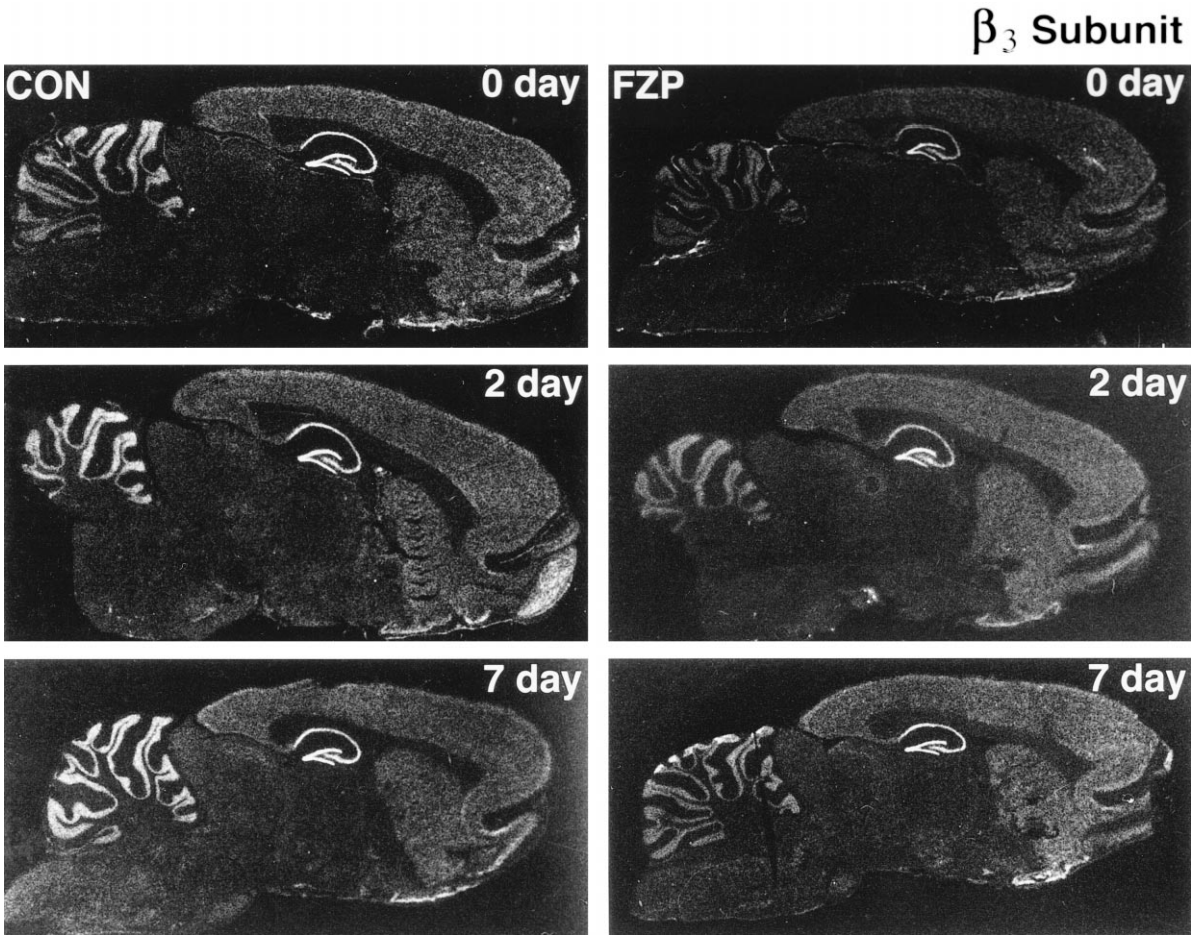

Fig. 2. Representative dark-field photomicrographs of autoradiographs of sagittal brain sections hybridized with an antisense [^{35}S]oligoprobe for the β3 subunit mRNA of the $GABA_A$ receptor. Sections (10 μm) were cut from one-week flurazepam-treated (FZP) rats killed immediately (day 0), two or seven days after ending oral drug administration and compared to sections from matched control rats (CON). Immediately after the end of treatment, there was a significant decrease in the relative gray level on autoradiographs localized over all principal cells in the hippocampus [CA1–CA3 pyramidal cells, polymorph (CA4) cells and granule cells], as well as the frontal cortex and caudate–putamen. The decreases in relative β3 subunit mRNA levels persisted in all regions two days after treatment. Seven days after stopping drug administration, there was an increase in mRNA levels in the CA3 region and in the frontal cortex (see Table 1).

developed as described previously.[66] Sections were stained with Cresyl Echt Violet (Fluka, Switzerland) for comparison with the hybridization signal.

Image analysis. Images on film were acquired using NIH Image Software (version 1.59) with a high-resolution CCD camera (Sierra Scientific, Sunnyvale, CA) mounted over a light box (Northern Light, Imaging Research, St Catherines, Ontario, Canada), which provided constant illumination. The overall illumination was adjusted so that the distribution of relative grey values, i.e. the number of pixels in the image as a function of grey value (0–256), fell within the limits of the system, typically within 30–220 gray value units. Once established, the settings remained constant for all images acquired within a particular experimental protocol. Images, acquired as described, were digitized and stored on an external Bernoulli disk (Iomega, Roy, UT) for later analysis using a Macintosh Quadra 950 computer.

Gray level measurements (per mm^2), which reflected mRNA levels, were made on digitized images by delimiting an area of interest, freehand, using predetermined criteria to define the region or subregion. For example, to measure the density on the image over the CA1 pyramidal cell layer, a perpendicular line was drawn from the outermost edge of the dentate gyrus (DG) at the hilus and from the lateral edge of the dorsal blade.[1] Within this defined area, the area delineated was limited to the innermost 90% of the total area to reduce the possible contribution of a signal from neighboring areas. Similar criteria were derived for each area measured. If necessary, e.g., to define cortical layers, the image was compared to an image of the Cresyl Echt Violet-stained section from the same rat. Background gray level was determined over the corpus collosum, a white matter area. All data are expressed as mean ± S.E.M. of the raw grey level values. Comparison of regional mRNA levels was made by ANOVA. Individual comparisons were made with orthogonal contrasts.

Dark-field images of silver grains detected over interneuron subclasses, defined by their location within the CA1 region [stratum oriens (SO), SO/stratum pyramidale (SP) border, SP or stratum radiatum (SR)], were used to detect the α1 mRNA level in coronal sections exposed to NTB-2 emulsion. Images were captured with a DAGE

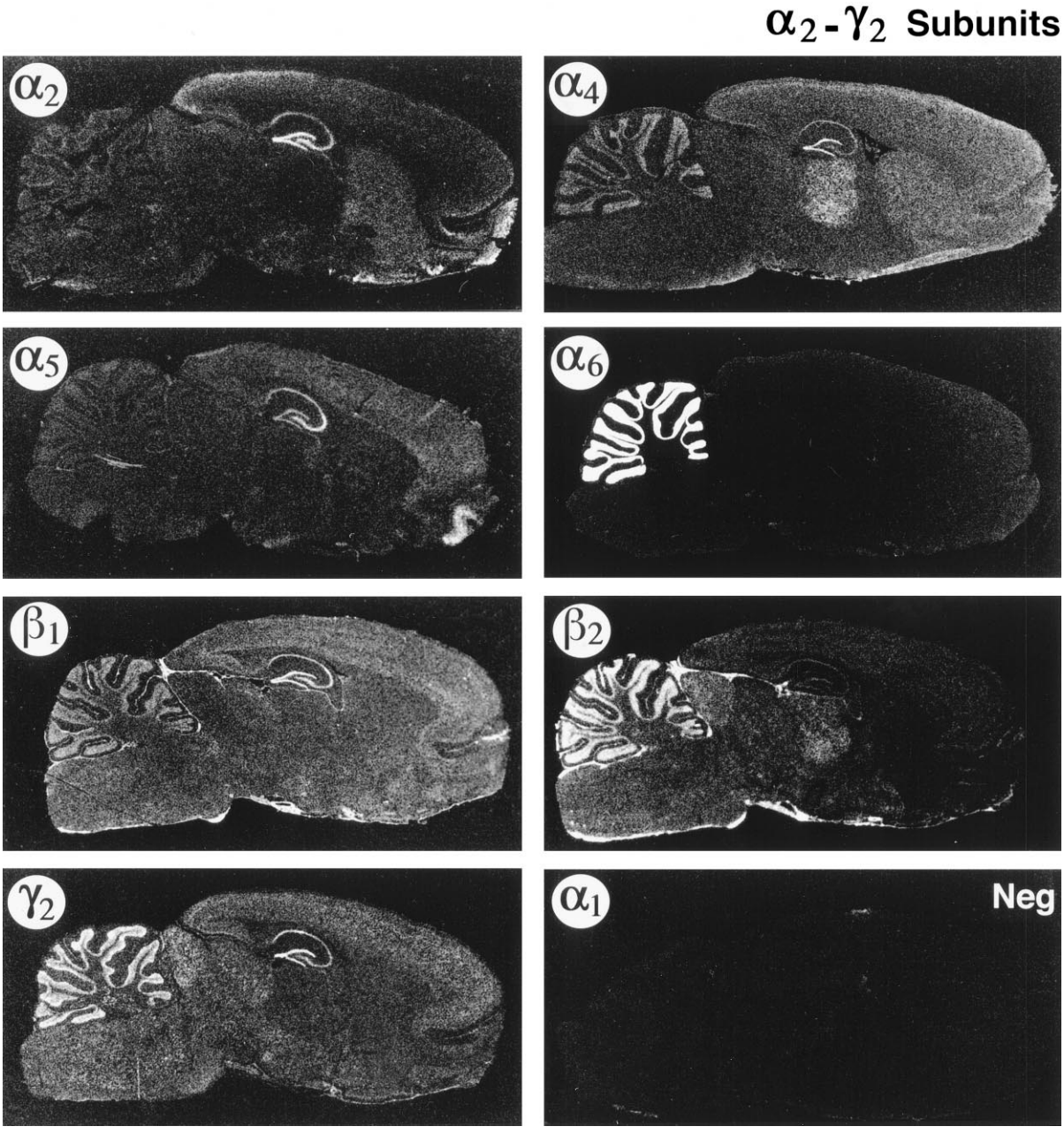

Fig. 3. Representative dark-field photomicrographs of autoradiographs of control rat sagittal brain sections hybridized with antisense [^{35}S]oligoprobes for the α2, α4, α5, α6, β1, β2 and γ2 subunit mRNAs of the $GABA_A$ receptor. All temporal groups for each [^{35}S]oligoprobe were processed identically as described in the Experimental Procedures. With the exception of β2 subunit mRNA levels, none of these $GABA_A$ receptor subunit mRNAs were regulated by one-week flurazepam treatment as indicated by a comparison of relative gray level values; therefore, additional representative autoradiographs of flurazepam-treated rat brains are not shown. There was a significant increase in the relative levels of the β2 mRNA two, but not seven, days after the end of drug administration localized to CA1 and CA3 pyramidal cells and dentate granule cells (see Table 1). A control section hybridized with the sense [^{35}S]oligoprobe for the α1 subunit mRNA, used as the negative (NEG) for α1 subunit mRNA levels, is also shown.

camera mounted on a Nikon Optiphot microscope (×125). Results obtained using the density threshold option of the image software were empirically determined to be equivalent to silver grain counting over bright-field images.[66] Relative grain density was defined as the "pixel" area covered by grains as a fraction of total pixel area of the interneuron soma of interest. Background density over the corpus collosum was subtracted from the total grain density to determine "α1 subunit-specific" grain density.

RESULTS

As illustrated in Figs 1–3, the relative distributions

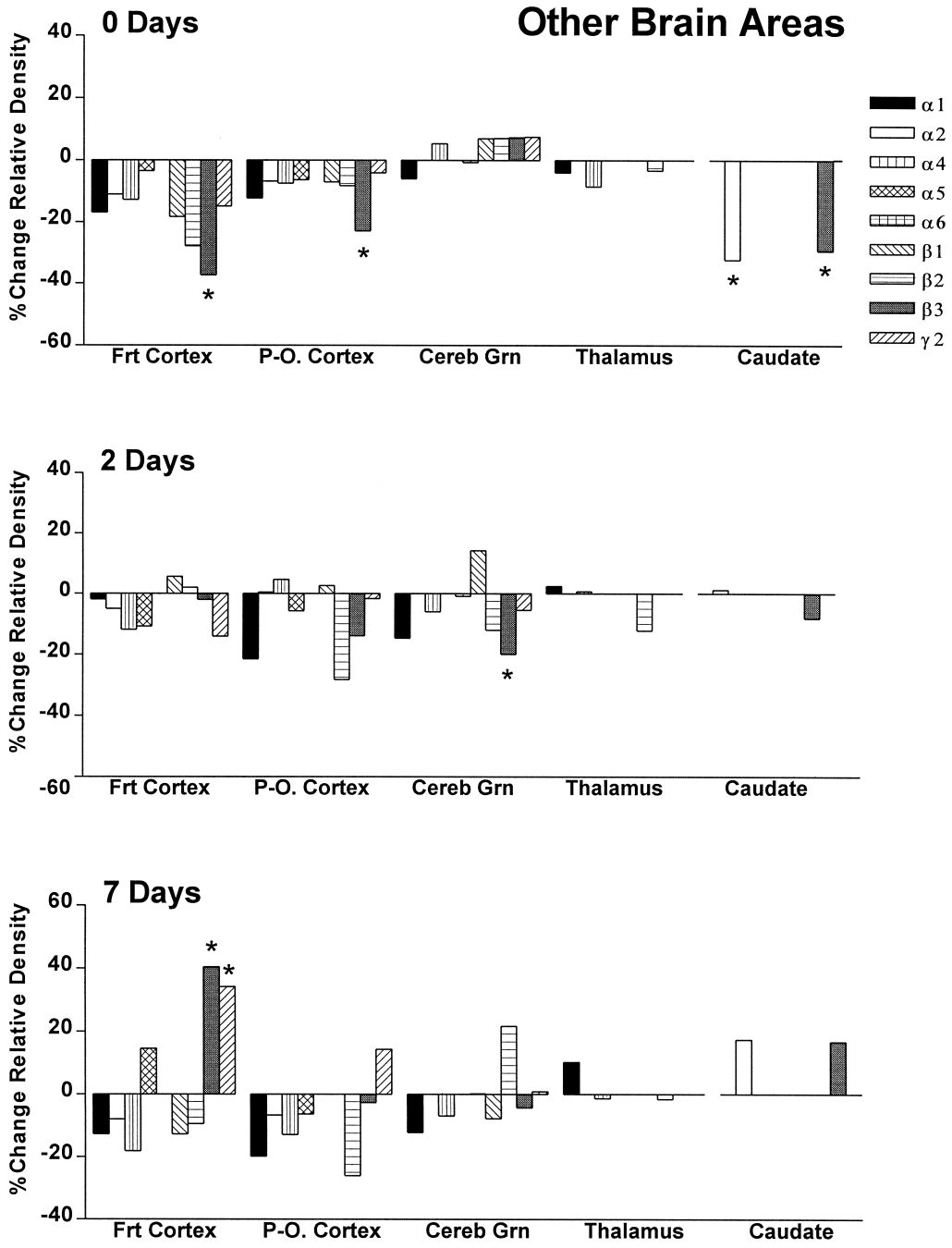

Fig. 4. Percentage change in relative density (gray level) on autoradiographs over principal hippocampal cell layers [CA1–CA3 pyramidal cells, polymorph cells (PC; formerly CA4 pyramidal cells) and DG cells]. Sections from rats killed immediately (day 0), two or seven days after ending oral flurazepam administration or their matched controls were handled identically and were batch processed according to the *in situ* hybridization procedures decribed in the Experimental Procedures. Flurazepam-treated and their matched control sections from each temporal group were exposed to the same film. Digitized images over autoradiographs were acquired with NIH image software, maintaining the same illumination intensity across all sections on each film. Statistical analyses were performed on raw gray level measurements (see Table 1). A significant decrease in α1 subunit mRNA levels, reflected in the percentage change in relative density immediately (day 0) after treatment, was localized to CA1 pyramidal cells and DG cells. The decrease only persisted in CA1 neurons two days after ending flurazepam treatment. A reduction in β3 subunit mRNA level, reflected in the change in relative density, was present in all principal hippocampal cells, immediately and two days after treatment. In contrast to the decreased β3 mRNA level, a significant increase in β2 subunit mRNA level was found in CA1, CA3 and granule cells two days after treatment. This trend persisted seven days after treatment. Whereas the γ2 subunit mRNA level was not significantly down-regulated immediately after stopping treatment, a similar trend toward an increase was seen two and seven days after flurazepam treatment. This biphasic trend was noted across all subunits, with the β1 subunit being the most notable exception.

Table 2. α1 Subunit messenger RNA level in interneurons in the CA1 region

	CA1 layer			
	SO	SO/SP border	SP	SR
Control	100.6 ± 3.1 (17)	115.5 ± 2.9 (15)	109.2 ± 2.2 (44)	94.4 ± 7.8 (5)
FZP treated	100.9 ± 3.2 (17)	101.0 ± 3.4 (7)	108.7 ± 2.0 (49)	95.7 ± 3.7 (7)
P value	0.94	0.01*	0.87	0.87

Mean (± S.E.M.) relative silver grain density. Number of cells/group is given in parentheses. FZP, flurazepam.
*Significant difference between control and flurazepam-treated groups, $P \leq 0.05$.

of the nine $GABA_A$ receptor subunit mRNAs evaluated were similar to those described by others.[49,52,75] The antisense [^{35}S]oligoprobe signal was specific to neurons and was very low to absent over white matter regions. Background labeling was uniform over RNase-treated sections and low over sections treated with sense strands for the α1 (Fig. 3) or β3 oligoprobes, as reported previously for each of the same oligoprobes.[75] All statistical analyses were made using raw gray level values (see Table 1). For comparison of the changes in the mRNA levels among the nine $GABA_A$ receptor subunits, the data in the text are expressed as percentages of the control mRNA level.

*α*1 *Subunit messenger RNA level*

Dark-field photomicrographs of α1 subunit mRNA distribution in parasagittal brain sections are shown in Fig. 1. As described previously, the relative distribution of the α1 subunit mRNA signal in rat hippocampal neurons was CA1 > DG ≥ CA3 > polymorph cells (formerly CA4). The α1 mRNA level was similar in the frontal, striatal and parietal cortices, and the thalamus. The α1 mRNA signal over the cerebellar granule cell layer was twofold greater than that over hippocampal granule cells.

The α1 subunit mRNA level was significantly decreased in the CA1 pyramidal cells (− 14.4%) and DG cells (− 19.5%) immediately (day 0) after the end of treatment (Table 1, Fig. 4). The significant decrease in α1 mRNA level persisted only in CA1 pyramidal cells two days after ending flurazepam treatment (− 11%) and was reversed seven days after ending treatment (− 5.8%). The decreased mRNA level in cortical areas did not reach significance at any time-point. There were no significant changes detected in α1 mRNA levels in other brain areas, i.e. the cerebellum and thalamus (Table 1).

In emulsion-coated sections, very densely labeled α1-positive interneurons were scattered throughout the neuropil and within and bordering the pyramidal cell layer.[22,34,66] Interneurons were classified according to their location within the hippocampal formation, i.e. SO, SO/SP border, SP and SR. Dark-field image analysis of silver grains over individual interneurons indicated a selective decrease in α1 mRNA level only in interneurons located at the SO/SP border (Table 2).

*α*2 *Subunit messenger RNA level*

The α2 subunit mRNA signal was moderate over CA1 pyramidal cells in comparison to CA3 pyramidal cells, polymorph cells and DG cells, the latter being of a relatively high density (Fig. 3). The level of α2 mRNA was relatively low in cortical areas and in the caudate–putamen, and negligible or absent in other brain areas in the parasagittal plane, i.e. the cerebellum, thalamus and collicular regions. The relatively large significant decrease in the α2 mRNA level in the caudate–putamen immediately after ending flurazepam treatment (− 32%) was reversed both two (+ 1.3%) and seven days (+ 17.4%) after ending treatment. No significant changes in α2 mRNA levels were found in any other brain areas at any time-point (Fig. 5).

*α*4 *Subunit messenger RNA level*

The pattern of the α4 mRNA signal in the hippocampus was relatively lower and more uniform in all pyramidal cell layers (Fig. 3) in comparison to that in DG cells, where it was twofold greater. α4 mRNA levels were relatively low in the cortex, cerebellar granule cells and thalamus. Chronic flurazepam treatment did not alter levels of α4 mRNA in any brain region at any of the time-points measured (Figs 4, 5).

*α*5 *Subunit messenger RNA level*

At the brain level examined, the α5 mRNA signal was relatively exclusive to the hippocampus and cortex (Fig. 3). The patterns of the α5 mRNA signal generated over hippocampal cells were similar to those described previously in emulsion-coated brain sections: CA3 > polymorph (CA4) cells > CA2 = CA1 ≫ DG.[66] The relative density in the cortex was similar to that in the dentate. As with the α4 subunit mRNA, there were no significant difference in α5 subunit mRNA levels post-flurazepam treatment (Figs 4, 5). However, there was a non-significant trend toward increased levels in DG cells (Fig. 4) and the frontal cortex (Fig. 5).

α6 Subunit messenger RNA level

As anticipated, the α6 subunit mRNA signal was intense and present exclusively in cerebellar granule cells (Fig. 3). Chronic flurazepam treatment had no effect on the level of α6 mRNA (Figs 4, 5).

β1 Subunit messenger RNA level

The level of β1 subunit mRNA was high in the hippocampus, with lower levels measurable in the cortex and in cerebellar granule cells (Fig. 3). The CA2 and the dentate region had very high levels of β1 subunit mRNA, CA2 pyramidal cells slightly higher than granule cells. Relatively high levels of β1 mRNA were also detected in CA1 and CA3 neurons, as well as polymorph cells. Chronic flurazepam treatment had no effect in regulating β1 subunit mRNA levels (Figs 4, 5).

β2 Subunit messenger RNA level

β2 Subunit mRNA levels were relatively low in the hippocampus (CA1 > CA2 = DG = CA3 = polymorph cells) and in the cortex, being most prominent in cerebellar granule cells (Fig. 3). The levels in the thalamus were somewhat higher than those in the CA1 region (Table 1). Though there was no effect of chronic flurazepam treatment immediately after the end of flurazepam treatment, β2 mRNA level was significantly up-regulated in CA1 (+ 31.2%) and CA3 (+ 37.2%) pyramidal cells and in DG cells (+ 29.2%) two days after ending treatment (Fig. 4). In the cortex, on the other hand, there was a non-significant trend toward a decrease in mRNA level immediately and two days after ending flurazepam treatment in the frontal and parieto-occipital cortices, respectively (Fig. 5, Table 1). There was no evidence of β2 subunit mRNA regulation in the other brain areas.

β3 Subunit messenger RNA level

The relative levels of β3 subunit mRNA in the hippocampus were as follows: DG ≫ polymorph cells > CA1 = CA3 > CA2 (Table 1, Fig. 2). The β3 mRNA level was relatively low in cortical areas and slightly lower in the caudate–putamen. Chronic flurazepam treatment induced time-dependent decreases in β3 subunit mRNA expression in several brain areas. Immediately after oral flurazepam administration was stopped, there were significant reductions in all pyramidal cell types (CA1, −24.0%; CA2, −14.8%; CA3, −18.4%) and in DG cells (−21.2%) (Fig. 4, Table 1). There was a large, significant reduction in β3 mRNA level in the frontal cortex (−37.3%), which did not reach significance in the parieto-occipital cortex (−22.8%). A significant reduction in β3 mRNA level (−29.2%) was also detected in the caudate–putamen, but not in the cerebellum (Fig. 5, Table 1). Two days after flurazepam was withdrawn, the reductions in β3 mRNA level persisted in the hippocampus. Seven days after ending flurazepam administration, several brain areas showed significant increases in β3 subunit mRNA levels, notably CA3 pyramidal cells (+17.8%) and frontal cortical cells (+34.2%). A similar trend was seen in other hippocampal cell groups and in the caudate–putamen, which did not reach significance (Fig. 5, Table 1).

γ2 Subunit messenger RNA level

The level of γ2 subunit mRNA was relatively uniform across the hippocampus, being somewhat higher in DG cells (Fig. 3). The level of γ2 subunit mRNA in cerebellar granule cells was comparable to that in hippocampal granule cells. The levels in the cortex were approximately 50% of those in the hippocampus. One-week flurazepam treatment had no measurable effect on γ2 mRNA expression in any brain area evaluated. None the less, as with the β3 mRNA expression, there was a trend toward increased expression seven days after flurazepam was withdrawn, which was significant in the frontal cortex (+34.2%; Fig. 5).

DISCUSSION

The hypothesis that a switch in subunit composition of the $GABA_A$ receptor may underlie benzodiazepine tolerance has received support from numerous studies. Nevertheless, reports have been contradictory, and with the exception of the α2 and γ1 mRNAs, changes in the levels of expression of each of the known α, β and γ subunit mRNAs have been detected following various *in vivo* benzodiazepine treatment regimens which result in anticonvulsant tolerance in rodents. For example, α1 subunit mRNA expression was decreased in the cerebral cortex[26,27,29,31,33,66,76] and hippocampus[29,31,66,76] following protracted benzodiazepine administration in some studies, and was unchanged in others.[26,33,46,85,86] Levels of γ2 or β3 subunit mRNA expression were down-regulated[33,55,86] or unchanged.[46] In contrast, other investigators reported up-regulation of subunit mRNAs.[27,31,47] One factor complicating the interpretation of findings is the inability to separate the molecular changes related to benzodiazepine tolerance from those associated with dependence. This divergence of findings may also be due to the benzodiazepine selected for chronic treatment, the dose, frequency and method of drug administration, and the time of analysis post-treatment. Moreover, changes in mRNA and protein expression following long-term benzodiazepine administration have been discretely localized[31,66] and may have gone undetected in previous northern blot or solution hybridization studies.[26–28,31,33,46,47,55,56,76,85] By focusing studies in the hippocampus, some of these problems can be

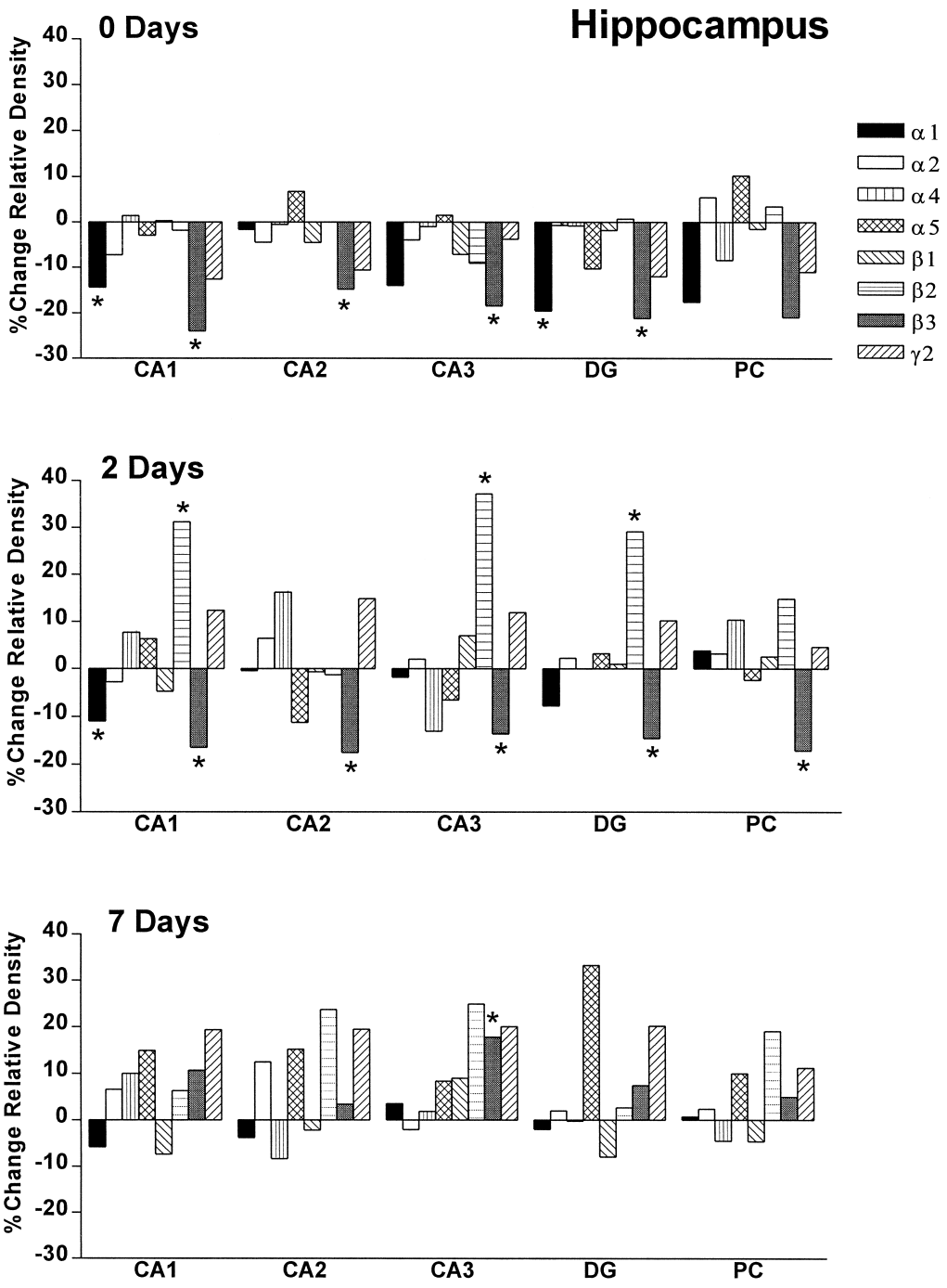

Fig. 5. Percentage change in relative density (gray level) on autoradiographs over brain areas with significant $GABA_A$ receptor subunit mRNA level (frontal cortex, parieto-occipital cortex, cerebellar granule cells, thalamus and caudate–putamen). Sections from rats killed immediately (day 0), two or seven days after ending oral flurazepam administration or their matched controls were handled identically and were batch processed according to the *in situ* hybridization procedures described in Experimental Procedures. Flurazepam-treated and their matching control sections from each temporal group were exposed to the same film. Digitized images over autoradiographs were acquired with NIH Image software, maintaining the same illumination intensity across all sections on each film. Statistical analyses were performed on raw gray level measurements (see Table 1). A significant decrease in β3 subunit mRNA level, reflected in the percentage change in relative gray level immediately after treatment, was localized to the frontal cortex. This decrease did not persist two days after ending treatment. There was a rebound increase in β3 subunit mRNA level seven days after ending flurazepam treatment. β3 Subunit mRNA level was also regulated biphasically in the caudate–putamen, but the rebound seven days after ending drug administration was not significant. Though γ2 subunit mRNA level was not significantly decreased immediately or two days after ending treatment, a significant increase in γ2 subunit mRNA level paralleled that of the β3 subunit mRNA in the frontal cortex. No other subunit mRNAs showed significant changes in their levels in any brain area measured.

circumvented, and there is an opportunity to relate changes in subunit mRNA expression to changes in $GABA_A$ receptor function in a well-defined neuronal population.

Relationship between $GABA_A$ receptor subunit messenger RNA expression and in vivo *tolerance*

The present *in situ* hybridization study was carried out on rats made tolerant to benzodiazepines *in vivo*, but not dependent. In our hippocampal model system, this treatment was also found to result in tolerance *in vitro*[78,83] and to significantly impair CA1 pyramidal cell GABAergic function.[77,78,80–84] Changes in the levels of α1, β2 and β3 subunit mRNAs were evident when tolerance was present (day 0 and two days), but had reversed when tolerance was absent (seven days). The discrete reduction in the level of α1 subunit mRNA in CA1 pyramidal cells two days after ending flurazepam treatment replicated our previous findings using silver grain analysis.[66] However, the time-course of the change in α1 subunit mRNA expression does not parallel that of benzodiazepine receptor down-regulation. In autoradiographic binding studies, significant, localized reductions in [^{3}H]flunitrazepam[10,67,68] and [^{3}H]zolpidem binding (unpublished observation) in the CA1 region of the hippocampus were transient, i.e. had reversed by two days after drug removal, though rats were still tolerant *in vivo* and *in vitro*. Conversely, GABA/benzodiazepine binding sites in the CA1 region remained "uncoupled" until tolerance was reversed.[10] Regulation of the α1 subunit has been implicated in mediating both receptor down-regulation and decreased allosteric coupling between GABA and benzodiazepine binding sites, yet its role is unresolved.[7,36,37,56,65] The benzodiazepine binding pocket is probably located at the interface of the α and γ subunits,[64] and recent studies using α/γ chimeras have also implicated two different domains on the γ subunit in regulating high-affinity benzodiazepine binding and allosteric coupling, respectively.[6] Other subunit mRNAs (α3, α4, α5, β1), including the γ2 subunit, have been shown to be regulated with acute, more protracted or more intense flurazepam treatments,[46,47,85,86] or following diazepam[27,31] or lorazepam[33] administration. Changes in γ2 subunit mRNA expression have been more closely linked with changes in benzodiazepine binding density.[85] None the less, changes in the mRNA expression of these subunits was not required to demonstrate anticonvulsant tolerance in *in vivo*[59,60] or in *in vitro* hippocampus[32,78,83] following flurazepam administration. In addition, unlike one-week oral flurazepam administration, other benzodiazepine treatment protocols which result in benzodiazepine tolerance and in a broader range of changes in $GABA_A$ receptor subunit mRNA expression may also be associated with benzodiazepine dependence.[31,73]

Studies of benzodiazepine site ligands which do not induce tolerance[28,31] may help clarify the conflicting findings of studies of mRNA expression following various chronic benzodiazepine treatments, since drugs which have limited tolerance-inducing potential, such as abercarnil[27] and imidazenil,[31] did not induce similar changes in mRNA expression. Chronic treatment with the α1-selective imidazopyridine, zolpidem, which also has a low tolerance liability, predictably down-regulated α1 subunit mRNA expression. However, since zolpidem has a negligible affinity for the α4 subunit, the up-regulation of α4 and β1 mRNA expression was unanticipated.[28] Holt *et al*.[27,28] proposed that some $GABA_A$ receptor subunit changes found following allosteric modulator treatments are the result of direct drug–receptor interactions and that changes in GABA tone may also affect steady-state levels of mRNA as the result of coordinated translation of $GABA_A$ subunit genes, found to be organized in clusters on human chromosomes (4p13–4q11: α2, α4, β1 and γ1; 5q32–5q33: α1, α6, β2 and γ2; 15q11–15q13: α5, β3, γ3).

Rebound overexpression of $GABA_A$ receptor subunit messenger RNAs

The lack of a significant change in the expression of the α5 subunit mRNA in the hippocampus and cortex,[66] at a time when rats are tolerant, replicates our previous findings. Expression of α5 mRNA, among other $GABA_A$ receptor subunit mRNAs, e.g., α3 and α4, was increased in whole brain or cortex following various chronic benzodiazepine treatments.[27,31,47] Conversely, a decrease in γ2 mRNA expression was reported following the same or other protocols.[31,85] Nevertheless, such subunit mRNA changes were not a prerequisite for *in vivo* or *in vitro* tolerance development during one-week flurazepam administration. Alternatively, the increase in β3 and γ2 mRNA in the frontal cortex and the trend toward the increased α5, β3 and γ2 mRNA expression in CA1, CA3 and DG cells, at a time when tolerance is no longer present *in vivo* (seven days), suggests that an α5β3γ2 receptor, a preferred hippocampal $GABA_A$ receptor subtype, (cf. Refs 41 and 44) might subsequently be overexpressed. Moreover, since dependence does not occur following one-week flurazepam treatment, the up-regulation of the β3 and γ2 subunit mRNAs in the frontal cortex likely represents a rebound, rather than a withdrawal phenomenon. A similar trend, increased expression seven days after ending flurazepam treatment, was shown for the majority of $GABA_A$ receptor subunit mRNAs examined. A role for biphasic switches in mRNA expression has not been established. Switches in the expression of certain subunit combinations occur during development,[19] perhaps reflecting both GABA's early neurotrophic role[69] and its subsequent role as an

inhibitory neurotransmitter in the hippocampus.[8] For example, in specific brain areas, α3, α5, β3 and γ2 subunit mRNAs peak during embryonic and early postnatal development, then rapidly decline. Thus, some of the switches in subunit subtypes detected following chronic benzodiazepine treatment may represent a coordinated control of the translation of specific $GABA_A$ receptor subtypes, analogous to the proposal of Holt *et al.*,[27,28] and may not be directly related to tolerance development.

Relationship between changes in subunit messenger RNA and protein levels

The localized decreases in the steady-state levels of α1 and β3 subunit mRNAs in the cortex and hippocampus of flurazepam-treated rats immediately after the drug was withdrawn was reflected in discrete, parallel changes in α1 and β3, but not α2, β1 or γ2, subunit protein in the same brain regions.[11] Quantitative immunohistochemical studies indicated a significant reduction in α1 subunit antibody staining density in the dendritic areas of CA1, CA3 and DG cells at the same time-point. β3 subunit antibody staining density was similarly decreased, but was more widespread in dentate areas. A comparison of the time-course of $GABA_A$ receptor subunit protein expression relative to mRNA expression will be needed to establish whether changes in protein expression persist at a time when tolerance is still detected in the hippocampus *in vitro*.[78,83] Preliminary experiments have suggested that the significant decrease in α1 subunit protein persists in the SO of the CA1 region, e.g., two days after ending flurazepam treatment (unpublished observations). Establishing a correlation between mRNA and protein levels is particularly important, since it is unknown whether changes in mRNA expression signal subsequent changes in protein translation or were regulated in a feedback manner. For example, exposure of chick cortical neurons to GABA resulted in a delayed decline in α1 subunit mRNA[4] and α1 subunit translation.[43] Whether a similar repression of mRNA expression occurs following benzodiazepine exposure is not yet known.[65] The strong correlation between changes in the levels of specific subunit mRNAs and subunit proteins in CA1 pyramidal cells suggests that the decreased levels of mRNA expression may represent a switch in $GABA_A$ receptor subunit composition and may be an important mechanism contributing to functional changes at the GABAergic postsynapse on CA1 pyramidal cells associated with benzodiazepine tolerance. Nevertheless, though the findings suggest that the β2 subunit could switch with the β3 subunit, no concomitant increases in α subunit mRNAs or proteins have been identified to suggest which α subunit might substitute for a decreased α1 subunit expression. Whether post-translational mechanisms may also relate to the decreased protein expression[36,37,56,65] or additionally contribute to changes in postsynaptic GABA function also requires further investigation.

Relationship between changes in $GABA_A$ receptor subunit messenger RNA expression and CA1 pyramidal cell function

The role of a switch in $GABA_A$ receptor subunits in mediating changes in GABAergic function is still uncertain. Decreased α1 subunit mRNA and protein expression in CA1 pyramidal cells[66] was associated with functional changes in these neurons following long-term benzodiazepine treatment.[77,80–82] The decreased potency of zolpidem to potentiate GABA currents in dissociated CA1 pyramidal cells of chronic diazepam-treated rats[32] and the reduced ability of zolpidem to potentiate the decay of CA1 pyramidal cell miniature inhibitory postsynaptic currents[83] suggests that the function of α1-containing $GABA_A$ receptor subtypes may be selectively impaired in the hippocampus of flurazepam-tolerant rats. The increased levels of β2 subunit mRNA in CA1 pyramidal cells two days after flurazepam administration was stopped, together with the decreased levels of β3 subunit mRNA and protein expression,[11] could account for the 2.5-fold decrease in $GABA_A$ agonist potency in the CA1 region of the hippocampus of flurazepam-tolerant rats.[78] One study indicated a similar 2.5-fold increase in GABA affinity in β3 vs β1 or β2 subunit-containing recombinant receptors,[15] though another report indicated no effect of β subunit substitution.[24] Conversely, a switch in the level of β2 and β3 mRNA subunits was also found in DG cells after chronic flurazepam treatment, yet in comparison to CA1 pyramidal neurons, this hippocampal cell group does not show a similar impairment in GABAergic function, i.e. a profound decrease in miniature inhibitory postsynaptic current amplitude or a reduction in unitary Cl^- channel conductance.[53,81] Reports have suggested that the β subunit of the $GABA_A$ receptor may have an important function to target receptors to the subsynaptic membrane and may play a role in regulating receptor localization or clustering during development or associated with plastic changes at GABAergic synapses.[12,51] The possible functional role of a switch in β subunits in benzodiazepine-tolerant rats remains uncertain.

Regulation of $GABA_A$ receptor subunit messenger RNA in hippocampal interneurons

Whole-cell electrophysiological studies in CA1 pyramidal cells in flurazepam-treated hippocampus also showed that spontaneous, but not miniature inhibitory postsynaptic current, frequency was reduced two days after the drug was withdrawn. These findings suggest that a presynaptic mechanism, a change in interneuron excitability, rather than a

change in GABA release mediated at GABAergic terminals, may be involved in the alteration in GABA function in tolerant rats.[81] The prominent localization of the α1 subunit protein on certain GABAergic interneuron populations[22,45] and its regulation in other principal hippocampal cell populations (Fig. 1, Table 1)[66] prompted the limited evaluation of α1 subunit mRNA levels over anatomically-defined interneuron subgroups. The reduction in α1 subunit mRNA levels in cells located at the SO/SP border (Table 2) is the first demonstration of the regulation of $GABA_A$ receptor mRNAs on presumptive interneurons, and warrants the further study of electrophysiologically, immunocytochemically and morphologically identified interneuron subpopulations[16,22,25,45] in the hippocampal CA1 region of chronic benzodiazepine-treated rats. Study of the regulation of other subunit mRNAs, e.g., the β2 subunit, on hippocampal interneurons and of the functional consequences of the regulation of mRNA expression on interneurons will also be important.

CONCLUSIONS

The levels of three (α1, β2 and β3) of nine $GABA_A$ receptor subunits were regulated as a function of time after ending one-week flurazepam treatment, a chronic benzodiazepine treatment which induces anticonvulsant tolerance but does not result in physical dependence. The same chronic benzodiazepine treatment was shown in previous electrophysiological studies to have profound consequences on hippocampal function and to induce benzodiazepine tolerance in the hippocampus *in vitro*. The findings of *in situ* hybridization studies suggested that changes in the levels of $GABA_A$ receptor mRNAs are discretely regulated in specific cortical regions, and in particular in hippocampal CA1 pyramidal neurons, with a pattern consistent with the appearance and disappearance of benzodiazepine anticonvulsant tolerance *in vivo* and *in vitro*. Exploratory studies suggested that postsynaptic $GABA_A$ receptors on selected interneuron populations were also regulated. Additional evidence has suggested that changes in the expression of specific $GABA_A$ receptor subunit mRNAs may mirror changes in the translation of their respective subunit proteins. Taken together, these findings suggest that regulation of α1, β2 and β3 subunits on $GABA_A$ receptors, in particular in the CA1 region of the hippocampus, may be a minimal requirement for initiating benzodiazepine anticonvulsant tolerance.

Acknowledgements—Portions of this work have appeared in abstract form [*Soc. Neurosci. Abstr.* (1994) **20,** 499]. This work was supported by National Institutes of Health grants R01-DA04075 and K02-DA00180. We thank Eugene Orlowski for technical assistance, and Drs X. J. Zeng and H. C. Rosenberg for helpful comments.

REFERENCES

1. Amaral D. G. and Witter M. P. (1995) Hippocampal formation. In *The Rat Nervous System* (ed. Paxinos G.), pp. 443–493. Academic, San Diego, CA.
2. Angelotti T. P. and Macdonald R. L. (1993) Assembly of $GABA_A$ receptor subunits: analysis of transient single-cell expression utilizing a fluorescent substrate/marker gene technique. *J. Neurosci.* **13,** 1418–1428.
3. Barnes E. M. Jr (1996) Use-dependent regulation of $GABA_A$ receptors. *Int. Rev. Neurobiol.* **39,** 53–76.
4. Baumgartner B. J., Harvey R. J., Darlison M. G. and Barnes E. M. Jr (1994) Developmental up-regulation and agonist-dependent down-regulation of $GABA_A$ receptor subunit mRNAs in chick cortical neurons. *Brain Res. molec. Brain Res.* **26,** 9–17.
5. Benke D., Fritschy J.-M., Trzeciak A., Bannwarth W. and Möhler H. (1994) Distribution, prevalence and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β-subunit variant. *J. biol. Chem.* **269,** 27,100–27,107.
6. Boileau A. J., Kucken A. M., Evers A. R. and Czajkowski C. (1998) Molecular dissection of benzodiazepine binding and allosteric coupling using chimeric γ-aminobutyric $acid_A$ receptor subunits. *Molec. Pharmac.* **53,** 295–303.
7. Brown M. J. and Bristow D. R. (1996) Molecular mechanisms of benzodiazepine-induced downregulation of $GABA_A$ receptor α1 subunit protein in rat cerebellar granule cells. *Br. J. Pharmac.* **118,** 1103–1110.
8. Chang C. C. A., Luntz-Leybman V., Evans J. E., Rotter A. and Frostholm A. (1995) Developmental changes in the expression of γ-aminobutyric $acid_A$/benzodiazepine receptor subunit mRNAs in the murine inferior olivary complex. *J. comp. Neurol.* **356,** 615–628.
9. >Chang Y., Wang R., Barot S. and Weiss D. A. (1996) Stoichiometry of a recombinant $GABA_A$ receptor. *J. Neurosci.* **16,** 5415–5424.
10. Chen S., Cox A. and Tietz E. I. (1995) Temporal regulation of $GABA_A$ receptor (GABAR) binding and coupling in flurazepam (FZP) tolerant rats. *Soc. Neurosci. Abstr.* **21,** 1590.
11. Chen S., Zeng X., Sieghart W. and Tietz E. I. (1996) Differential changes in $GABA_A$ receptor (GABAR) α1, α2, β1, β3 and γ2 subunit proteins in flurazepam (FZP) tolerant rats. *Soc. Neurosci. Abstr.* **22,** 1285.
12. Connolly C. N., Wooltorton J. R. A., Smart T. and Moss S. J. (1996) Subcellular localization of γ-aminobutyric type A receptors is determined by β subunits. *Proc. natn. Acad. Sci. U.S.A.* **93,** 9899–9904.
13. Davies P. A., Hanna M. C., Hales T. G. and Kirkness E. (1997) Insensitivity to anaesthetic agents conferred by a class of $GABA_A$ receptor subunits. *Nature* **385,** 820–823.
14. Doble A. and Martin I. L. (1992) Multiple benzodiazepine receptors: no reason for anxiety. *Trends pharmac. Sci.* **13,** 76–81.
15. Ducic I., Caruncho H. J., Zhu W. J., Vicini S. and Costa E. (1995) γ-Aminobutyric acid gating of Cl^- channels in recombinant $GABA_A$ receptors. *J. Pharmac. exp. Ther.* **272,** 438–445.
16. Freund T. F. and Buzsaki G. (1996) Interneurons of the hippocampus. *Hippocampus* **6,** 347–470.
17. Fritschy J.-M., Benke D., Mertens S., Oertel W. H., Bachi T. and Möhler H. (1992) Five subtypes of type A GABA receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. *Proc. natn. Acad. Sci. U.S.A.* **89,** 6726–6730.

18. Fritschy J.-M. and Möhler H. (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. comp. Neurol.* **359,** 154–194.
19. Fritschy J.-M., Paysan J., Enna A. and Möhler H. (1994) Switch in the expression of rat $GABA_A$ receptor subtypes during postnatal development: an immunohistochemical study. *J. Neurosci.* **14,** 5302–5324.
20. Gallager D. W., Malcolm A. B., Anderson S. A. and Gonsalves S. F. (1985) Continuous release of diazepam: electrophysiological, biochemical and behavioral consequences. *Brain Res.* **342,** 26–36.
21. Gallager D. W., Marley R. J. and Hernandez T. D. (1991) Biochemical and electrophysiological mechanisms underlying benzodiazepine tolerance and dependence. In *The Biological Bases of Drug Tolerance and Dependence* (ed. Pratt J.), pp. 49–70. Academic, New York.
22. Gao B. and Fritschy J. M. (1994) Selective allocation of $GABA_A$ receptors containing the α1 subunit to neurochemically distinct subpopulations of rat hippocampal interneurons. *Eur. J. Pharmac.* **6,** 837–853.
23. Gao B., Fritschy J. M., Benke D. and Möhler H. (1993) Neuron-specific expression of $GABA_A$-receptor subtypes: differential association of the α1- and α3-subunits with serotonergic and GABAergic neurons. *Neuroscience* **54,** 881–892.
24. Hadingham K. L., Wingrove P. B., Wafford K. A., Bain C., Kemp J. A., Palmer K. J., Wilson A. W., Wilcox A. S., Sikela J. M., Ragan C. I. and Whiting P. J. (1993) Role of the β subunit in determining the pharmacology of human γ-aminobutyric acid type A receptors. *Molec. Pharmac.* **44,** 1211–1218.
25. Hájos N. and Mody I. (1997) Synaptic communication among hippocampal interneurons: properties of spontaneous IPSCs in morphologically-identified cells. *J. Neurosci.* **17,** 8427–8442.
26. Heninger C., Saito N., Tallman J. F., Garrett K. M., Vitek M. P., Duman R. S. and Gallager D. W. (1990) Effects of continuous diazepam administration on $GABA_A$ subunit mRNA in rat brain. *J. molec. Neurosci.* **2,** 101–107.
27. Holt R. A., Bateson A. N. and Martin I. L. (1996) Chronic treatment with diazepam or abecarnil differently affects the expression of $GABA_A$ receptor subunit mRNAs in the rat cortex. *Neuropharmacology* **35,** 1457–1463.
28. Holt R. A., Bateson A. N. and Martin I. L. (1997) Chronic zolpidem treatment alters GABA(A) receptor mRNA levels in the rat cortex. *Eur. J. Pharmac.* **329,** 129–132.
29. Huang X., Ferencak W. J., Chen S. and Tietz E. I. (1994) Temporal regulation of $GABA_A$ receptor α and β subunit mRNA *in situ* after chronic flurazepam treatment. *Soc. Neurosci. Abstr.* **20,** 499.
30. Hutchinson M. A., Smith P. F. and Darlington C. L. (1996) The behavioral and neuronal effects of chronic administration of benzodiazepine anxiolytic and hypnotic drugs. *Prog. Neurobiol.* **49,** 73–97.
31. Impagnatiello F., Persold C., Longone P., Caruncho H., Fritschy J. M., Costa E. and Guidotti A. (1996) Modifications of γ-aminobutyric $acid_A$ receptor subunit expression in rat neocortex during tolerance to diazepam. *Molec. Pharmac.* **49,** 822–831.
32. Itier V., Granger P., Perrault G., Depoortere H., Scatton B. and Avenet P. (1996) Protracted treatment with diazepam reduces benzodiazepine receptor-mediated potentiation of γ-aminobutyric acid-induced currents in dissociated rat hippocampal neurons. *J. Pharmac. exp. Ther.* **279,** 1092–1099.
33. Kang L. and Miller L. G. (1991) Decreased $GABA_A$ receptor subunit mRNA concentrations following chronic lorazepam administration. *Br. J. Pharmac.* **103,** 1283–1287.
34. Khrestchatisky M., MacLennan A. J., Chiang M.-Y., Xu W., Jackson M. B., Brecha N., Sternini C., Olsen R. W. and Tobin A. J. (1989) A novel α subunit in rat brain $GABA_A$ receptors. *Neuron* **3,** 745–753.
35. Khrestchatisky M., MacLennan A. J., Tillakaratne N. J. K., Chiang M.-Y. and Tobin A. J. (1991) Sequence and regional distribution of the mRNA encoding the α2 polypeptide of rat γ-aminobutyric $acid_A$ receptors. *J. Neurochem.* **56,** 1717–1722.
36. Klein R. L., Mascia M. P., Harkness P. C., Hadingham K. L., Whiting P. J. and Harris R. A. (1995) Regulation of allosteric coupling and function of stably expressed γ-aminobutyric acid $(GABA)_A$ receptors by chronic treatment with $GABA_A$ and benzodiazepine agonists. *J. Pharmac. exp. Ther.* **274,** 1484–1492.
37. Klein R. L., Whiting P. J. and Harris R. A. (1994) Benzodiazepine treatment causes uncoupling of recombinant $GABA_A$ receptors expressed in stably transfected cells. *J. Neurochem.* **63,** 2349–2351.
38. Lüddens H., Pritchett D. B., Kohler M., Killisch I., Keinanen K., Monyer H., Sprengel R. and Seeburg P. H. (1990) Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. *Nature* **346,** 648–651.
39. Macdonald R. L. and Olsen R. W. (1994) $GABA_A$ receptor channels. *A. Rev. Neurosci.* **17,** 569–602.
40. Malherbe P., Sigel E., Baur R., Persohn E., Richards J. G. and Möhler H. (1990) Functional expression and sites of gene transcription of a novel α subunit of the $GABA_A$ receptor in rat brain. *Fedn Eur. biochem. Socs Lett.* **260,** 261–265.
41. McKernan R. M. and Whiting (1996) Which $GABA_A$-receptor subtypes really occur in the brain? *Trends Neurosci.* **19,** 139–143.
42. Miller L. G., Greenblatt D. J., Barnhill J. G. and Shader R. I. (1988) Chronic benzodiazepine administration. I. Tolerance is associated with benzodiazepine receptor downregulation and decreased γ-aminobutyric acid A receptor function. *J. Pharmac. exp. Ther.* **246,** 170–176.
43. Miranda J. D. and Barnes E. M. Jr (1997) Repression of γ-aminobutyric acid Type A receptor α1 polypeptide biosynthesis requires chronic agonist exposure. *J. biol. Chem.* **272,** 16,288–16,294.
44. Möhler H., Knoflach F., Paysan J., Motejlek K., Benke D., Lüscher B. and Fritschy J.-M. (1995) Heterogeneity of $GABA_A$-receptors: cell specific expression, pharmacology, and regulation. *Neurochem. Res.* **20,** 631–636.
45. Nusser Z., Sieghart W., Benke D., Fritschy J. M. and Somogyi P. (1996) Differential synaptic localization of two major gamma-aminobutyric acid type A receptor alpha subunits on hippocampal pyramidal cells. *Proc. natn. Acad. Sci. U.S.A.* **93,** 11939–11944.
46. O'Donovan M. C., Buckland P. R. and McGuffin P. (1992) Levels of $GABA_A$ receptor subunit mRNA in rat brain following flurazepam treatment. *J. Psychopharmac.* **6,** 364–369.
47. O'Donovan M. C., Buckland P. R., Spurlock G. and McGuffin P. (1992) Bi-directional changes in the levels of messenger RNAs encoding γ-aminobutyric acid A receptor α subunits after flurazepam treatment. *Eur. J. Pharmac.* **226,** 335–341.
48. Olsen R. W., McCabe R. T. and Wamsley J. K. (1990) $GABA_A$ receptor subtypes: autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in rat central nervous system. *J. chem. Neuroanat.* **3,** 59–76.
49. Olsen R. W. and Tobin A. J. (1990) Molecular biology of $GABA_A$ receptors. *Fedn Proc. Fedn Am. Socs exp. Biol.* **4,** 1469–1480.
50. Paxinos G. and Watson C. (1986) *The Rat Brain in Stereotaxic Coordinates*, 2nd edn. Academic, Orlando, FL.
51. Perez-Velazquez J. L. and Angelides K. J. (1993) Assembly of $GABA_A$ receptor subunits determines sorting and localization in polarized cells. *Nature* **361,** 457–460.
52. Persohn E., Malherbe P. and Richards J. G. (1992) Comparative molecular neuroanatomy of cloned $GABA_A$ receptor subunits in the rat CNS. *J. comp. Neurol.* **326,** 193–216.

53. Poisbeau P., Williams S. J. and Mody I. (1997) Silent $GABA_A$ synapses during flurazepam withdrawal are region specific in the hippocampal formation. *J. Neurosci.* **17,** 3467–3475.
54. Poncer J. C., Durr R., Gähwiler B. H. and Thompson S. M. (1996) Modulation of synaptic $GABA_A$ receptor function by benzodiazepines in area CA3 of rat hippocampal slice cultures. *Neuropharmacology* **35,** 1169–1179.
55. Primus R. J. and Gallager D. W. (1992) $GABA_A$ receptor subunit mRNA levels are differentially influenced by chronic FG 7142 and diazepam exposure. *Eur. J. Pharmac.* **226,** 21–28.
56. Primus R. J., Yu J., Xu J., Hartnett C., Meyyappan M., Kostas C., Ramabhadran T. V. and Gallager D. W. (1996) Allosteric uncoupling after chronic benzodiazepine exposure of recombinant γ-aminobutyric $acid_A$ receptors expressed in Sf9 cells: ligand efficacy and subtype selectivity. *J. Pharmac. exp. Ther.* **276,** 882–890.
57. Pritchett D. B., Sontheimer H., Shivers B. D., Ymer S., Kettenmann H., Schofield P. R. and Seeburg P. H. (1989) Importance of a novel GABA(A) receptor subunit for benzodiazepine pharmacology. *Nature* **338,** 582–585.
58. Ramsey-Williams V. A., Wu Y. and Rosenberg H. C. (1994) Comparison of anticonvulsant tolerance, cross tolerance and benzodiazepine receptor binding following chronic treatment with diazepam or midazolam. *Pharmac. Biochem. Behav.* **48,** 722–765.
59. Rosenberg H. C. (1995) Differential expression of benzodiazepine anticonvulsant cross-tolerance according to time following flurazepam or diazepam treatment. *Pharmac. Biochem. Behav.* **51,** 363–368.
60. Rosenberg H. C., Tietz E. I. and Chiu T. H. (1985) Tolerance to the anticonvulsant action of benzodiazepines: relationship to decreased receptor density. *Neuropharmacology* **24,** 639–644.
61. Shivers B. D., Killisch I., Sprengel R., Sontheimer H., Kohler M., Schofield P. R. and Seeburg P. H. (1989) Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. *Neuron* **3,** 327–337.
62. Sieghart W. (1995) Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharmac. Rev.* **47,** 181–234.
63. Sigel E., Baur R., Trube G., Möhler H. and Mahlerbe P. (1990) The effect of subunit composition of rat brain $GABA_A$ receptors on channel function. *Neuron* **5,** 703–711.
64. Smith G. B. and Olsen R. W. (1995) Functional domains of $GABA_A$ receptors. *Trends pharmac. Sci.* **16,** 167–168.
65. Tehrani M. H. J. and Barnes E. M. Jr (1997) Sequestration of γ-aminobutyric $acid_A$ receptors on clathrin-coated vesicles during chronic benzodiazepine administration *in vivo*. *J. Pharmac. exp. Ther.* **283,** 384–390.
66. Tietz E. I., Huang X., Weng X., Rosenberg H. and Chiu T. H. (1994) Expression of α1, α5, and γ2 $GABA_A$ receptor subunit mRNAs measured *in situ* in rat hippocampus and cortex following chronic flurazepam administration. *J. molec. Neurosci.* **4,** 277–292.
67. Tietz E. I. and Rosenberg H. C. (1988) Behavioral measurement of benzodiazepine tolerance and GABAergic subsensitivity in the substantia nigra pars reticulata. *Brain Res.* **438,** 41–51.
68. Tietz E. I., Rosenberg H. C. and Chiu T. H. (1986) Autoradiographic localization of benzodiazepine receptor downregulation. *J. Pharmac. exp. Ther.* **236,** 284–292.
69. Valeyev A. Y., Cruciani R. A., Lange G. D., Smallwood V. S. and Barker J. L. (1993) Cl^- channels are randomly activated by continuous GABA secretion in cultured rat hippocampal neurons. *Neurosci. Lett.* **155,** 199–203.
70. Verdoorn T. A., Draguhn A., Ymer S., Seeburg P. H. and Sakmann B. (1990) Functional properties of recombinant rat $GABA_A$ receptors depend upon subunit composition. *Neuron* **4,** 919–928.
71. Vicini S. (1991) Pharmacological significance of the structural heterogeneity of the $GABA_A$ receptor–chloride ion channel complex. *Neuropsychopharmacology* **4,** 9–15.
72. Williamson R. E. and Pritchett D. B. (1994) Levels of benzodiazepine receptor subtypes and $GABA_A$ receptor α-subunit mRNA do not correlate during development. *J. Neurochem.* **63,** 413–418.
73. Wilson M. A. and Gallager D. W. (1988) Ro 15-1788-induced seizures in rats continually exposed to diazepam for prolonged periods. *Epilepsy Res.* **2,** 14–19.
74. Wisden W., Herb A., Wieland H., Keinänen K., Lüddens H. and Seeburg P. H. (1991) Cloning, pharmacological characteristics and expression pattern of the rat $GABA_A$ receptor α_4 subunit. *Fedn Eur. biochem. Socs Lett.* **289,** 227–230.
75. Wisden W., Laurie D. J., Monyer H. and Seeburg P. H. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, and mesencephalon. *J. Neurosci.* **12,** 1040–1062.
76. Wu Y., Rosenberg H. C., Chiu T. H. and Zhao T. J. (1994) Subunit- and brain region-specific reduction of $GABA_A$ receptor subunit mRNAs during chronic treatment of rats with diazepam. *J. molec. Neurosci.* **5,** 105–120.
77. Xie X.-H. and Tietz E. I. (1991) Chronic benzodiazepine treatment of rats induces reduction of paired-pulse inhibition in CA1 region of *in vitro* hippocampal slices. *Brain Res.* **561,** 69–76.
78. Xie X.-H. and Tietz E. I. (1992) Reduction in potency of selective $GABA_A$ agonists and diazepam in CA1 region of *in vitro* hippocampal slices from chronic flurazepam treated rats. *J. Pharmac. exp. Ther.* **262,** 204–210.
79. Ymer S., Schofield P. R., Draguhn A., Werner P., Kohler M. and Seeburg P. H. (1989) $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. *Eur. molec. Biol. Org. J.* **8,** 1665–1670.
80. Zeng X. and Tietz E. I. (1994) Impairment of feedforward inhibition in CA1 region of hippocampus after chronic benzodiazepine treatment. *Neurosci. Lett.* **173,** 40–44.
81. Zeng X. and Tietz E. I. (1995) Decreased amplitude and frequency of spontaneous IPSCs recorded in hippocampal CA1 pyramidal neurons after chronic flurazepam (FZP) treatment. *Soc. Neurosci. Abstr.* **21,** 1589.
82. Zeng X. and Tietz E. I. (1997) Depression of both early and late monosynaptic inhibitory postsynaptic potentials in hippocampal CA1 neurons following chronic benzodiazepine administration. *Synapse* **25,** 125–136.
83. Zeng X. J. and Tietz E. I. (1998) Benzadiazepine tolerance at GABAergic synapses on hippocampal CA1 pyramidal cells. *Synapse* (in press).
84. Zeng X., Xie X.-H. and Tietz E. I. (1995) Reduction of GABA-mediated IPSPs in hippocampal CA1 pyramidal neurons following oral flurazepam administration. *Neuroscience* **66,** 87–99.
85. Zhao T., Chiu T. H. and Rosenberg H. C. (1994) Reduced expression of γ-aminobutyric acid Type A/benzodiazepine receptor γ2 and α5 subunit mRNAs in brain regions of flurazepam-treated rats. *Molec. Pharmac.* **45,** 657–663.
86. Zhao T., Chiu T. H. and Rosenberg H. C. (1994) Decreased expression of γ-aminobutyric acid Type A/benzodiazepine receptor β subunit mRNAs in flurazepam-tolerant rats. *J. molec. Neurosci.* **5,** 181–192.

(*Accepted* 1 *September* 1998)