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Expression of α_1, α_5, and γ_2 $GABA_A$ Receptor Subunit mRNAs Measured *In Situ* in Rat Hippocampus and Cortex Following Chronic Flurazepam Administration**

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Contents

Abstract

Prolonged benzodiazepine treatment of rats results in anticonvulsant tolerance in vivo. Studies of in vitro hippocampal slices following 1 wk flurazepam administration show reduced GABA-mediated inhibition in the CA1 region, and a decrease in $GABA_A$ agonist and benzodiazepine potency to inhibit CA1 pyramidal cell-

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evoked responses. To investigate the molecular basis of benzodiazepine tolerance in the hippocampus, *in situ* hybridization techniques were used to evaluate the expression of the mRNAs for the α_1, α_5, and γ_2 subunits of the $GABA_A$ receptor in the hippocampal formation and frontal cortex of chronic flurazepam-treated rats. A discretely localized decrease in α_1, but not α_5 or γ_2 mRNA expression was found in the CA1 region (35–40%) and in layers II–III and IV of cortex (50–60%) 2 d after cessation of flurazepam treatment. The decrease in the expression of α_1 subunit mRNA in cortex is similar to that reported following other chronic benzodiazepine treatment regimens. This is the first report of a reduction in α_1 subunit mRNA expression in the hippocampal formation.

Index Entries: Benzodiazepines; flurazepam; GABA; hippocampus; *in situ* hybridization; tolerance.

Introduction

The sedative and anticonvulsant actions of the benzodiazepines are related to their ability to potentiate GABA-mediated neurotransmission (Haefely, 1985) through an increase in the frequency of GABA-gated chloride channel opening (Barker and Mathers, 1981). Prolonged benzodiazepine administration in man and in several animal species results in tolerance to these actions and many studies have established that benzodiazepine tolerance is related to an impairment of GABA-mediated inhibitory neurotransmission (Gallager et al., 1991). Nonetheless, the specific changes and sequence of molecular events that occur at GABAergic synapses in association with benzodiazepine tolerance have not been delineated.

Study of the neurophysiology and pharmacology of the hippocampus in benzodiazepine-tolerant rats has provided a useful model to study the synaptic mechanisms of benzodiazepine tolerance. Oral administration of flurazepam that results in benzodiazepine tolerance in vivo (Rosenberg et al., 1991) decreases GABA-mediated feedforward and recurrent inhibition in the hippocampal CA1 pyramidal cell region (Xie and Tietz, 1991; Zeng et al., 1994). Decreased GABA inhibition was associated with a 50% reduction in GABA-mediated inhibitory postsynaptic potentials (IPSPs) (Zeng et al., 1994). Although presynaptic mechanisms may contribute to the neurophysiological changes measured in the hippocampus after prolonged flurazepam administration, the evidence suggests that a functional alteration of the postsynaptic $GABA_A$ receptor plays an important role. For example, 1 and 4 wk flurazepam administration downregulated [^{3}H]flunitrazepam binding sites in several regions of rat brain, including hippocampus (Tietz et al., 1986). Likewise, a decrease in benzodiazepine binding in cortex and hippocampus of mice was reported following chronic lorazepam treatment (Miller et al., 1988). Further, the potency of superfused $GABA_A$, but not $GABA_B$, agonists to inhibit CA1 pyramidal cell-evoked responses was reduced in in vitro hippocampal slices of flurazepam treated rats (Xie and Tietz, 1992). Tolerance to diazepam, i.e., a decrease in diazepam's potency to potentiate GABA agonist responses, was also measured in this model (Xie and Tietz, 1992). Although GABA potency was reduced in the CA1 region, CA3 neurons did not exhibit reduced sensitivity to GABA after chronic diazepam administration (Lista et al., 1990).

The $GABA_A$ receptor, a hetero-oligomeric protein with five or fewer subunits, comprises a chloride ionophore, a GABA binding site, and several binding sites for allosteric modulators of GABA function, including the benzodiazepines (Olsen and Tobin, 1990; Sigel et al., 1990; Burt and Kamatchi, 1991; Sieghart, 1992). With the cloning of multiple $GABA_A$ receptor subunit cDNAs, it has become possible to investigate the molecular basis for benzodiazepine tolerance (Schofield et al., 1987; Khrestchatisky et al., 1989; Shivers et al., 1989; Malherbe et al., 1990; Burt and Kamatchi, 1991). Although the composition of native $GABA_A$ receptor

subtypes is unknown, they are likely to have the form, $2\alpha_{1-6}$, $2\beta_{1-3}$, γ_2. Studies of recombinantly expressed $GABA_A$ receptors indicate that the γ_2 subunit confers benzodiazepine sensitivity (Pritchett et al., 1989a; Sigel et al., 1990; Angelotti and Macdonald, 1993). At least 50% of immunoprecipitated $GABA_A$ receptors contain a γ_2 subunit (Benke et al., 1991), which in association with an α subunit is required for benzodiazepine binding (Pritchett et al., 1988, 1989a,b; Pritchett and Seeburg, 1990). A change in the expression of the genes encoding the various subunits of the $GABA_A$ receptor, and thus a change in subunit composition, has been proposed by several investigators as one mechanism for the regulation of $GABA_A$ receptor function after chronic benzodiazepine administration (Heninger et al., 1990; Kang and Miller, 1991; Primus and Gallager, 1992; Zhao et al., 1994).

$GABA_A$ receptor subunit variants are heterogeneously distributed throughout the brain and are in particularly high abundance in the hippocampus and cortex (Levitan et al., 1988; Sequier et al., 1988; Wisden et al., 1988, 1992; Shivers et al., 1989; Benke et al., 1991). In spite of reports of the modification of hippocampal neuronal function and pharmacology by chronic benzodiazepine treatment (Tietz et al., 1986; Miller et al., 1988; Xie and Tietz, 1991, 1992; Zeng et al., 1993, 1994), studies of $GABA_A$ subunit mRNAs using Northern blot analysis have often failed to reveal changes in the hippocampus (Heninger et al., 1990; Kang and Miller, 1991; Primus and Gallager, 1992). For example, α_1 and γ_2 (but not β_1) mRNAs were decreased in cortex, but not hippocampus or cerebellum, after 3 wk diazepam administration (Heninger et al., 1990; Primus and Gallager, 1992). Similarly after 2 wk, but not 1 wk, lorazepam administration in mice α_1 and γ_2 mRNAs were decreased in cortex, but not hippocampus or cerebellum (Kang and Miller, 1991). In this investigation, *in situ* hybridization histochemistry, which may be a more sensitive measure of regional variations in subunit composition, was used to examine the expression of mRNAs encoding the α_1, α_5, and γ_2 $GABA_A$ receptor subunits in the cerebral cortex and hippocampus of rats following 1 wk oral administration of flurazepam.

Materials and Methods

Chronic Flurazepam Treatment

Male Sprague-Dawley rats were administered flurazepam HCl (pH 6.3) orally for 1 wk in a 0.02% saccharin solution as their sole source of drinking water (Tietz et al., 1986; Tietz and Rosenberg, 1988; Rosenberg et al., 1991; Xie and Tietz, 1991, 1992; Zeng et al., 1993). The dose was adjusted daily according to the rats' body weight and fluid consumption to offer up to 100 mg/kg for 3 d and 150 mg/kg for 4 d. For comparison with electrophysiological data (Xie and Tietz, 1991, 1992; Zeng et al., 1993), rats were sacrificed 2 d after the end of treatment. Saccharin water was offered 2 d before drug administration and for 2 d after the termination of drug treatment. Pair-handled controls received saccharin vehicle for the entire period. Only rats that consumed a weekly average of ≥100 mg/kg/d were used in the study. The actual average weekly dose, 120.3 ± 2.3 mg/kg/d, was in the range of that reported previously (Tietz and Rosenberg, 1988; Xie and Tietz, 1991, 1992). Benzodizepine brain levels achieved during 1 wk flurazepam administration, expressed in diazepam equivalents (135.4 ± 14.2 ng/g hippocampus) (Xie and Tietz, 1991), are similar to or less than those achieved with other chronic benzodiazepine treatments (Gallager et al., 1985; Miller et al., 1988). Rats treated with this regimen never exhibit spontaneous, and rarely exhibit precipitated, withdrawal effects (Tietz and Rosenberg, 1988).

In Situ Hybridization Histochemistry

Methods for *in situ* hybridization histochemistry of α_1, α_5, and γ_2 mRNAs were modified from Siegel (1988, 1989) and Wisden et al. (1988,

1992). All flurazepam-treated and control tissue was handled in parallel throughout the experiments.

Oligonucleotide Probes

Oligonucleotide probes (45-mers) (Oligos Etc., Inc., Wilsonville, OR) were complimentary to rat cDNA subunit sequences (Wisden et al., 1992) (α_1: amino acid residues 342–356 [Khrestchatisky et al., 1989]; α_5: 355–369 [Malherbe et al., 1990; α_4 according to Khrestchatisky et al., 1989]; γ_2: 338–352 [Shivers et al., 1989]). Probes were 3' end-labeled with [^{35}S]dATP (New England Nuclear, Boston, MA, 1432 Ci/mmol) using terminal deoxynucleotidyl transferase according to Siegel (1989).

Tissue Preparation

Rats were sacrificed and the brains were rapidly dissected and frozen in isopentane cooled in an acetone/dry ice bath. Frozen sections (10 μ*M*) were cut (–12 to –14°C) at the level of the dorsal hippocampus (bregma –3.8; Paxinos and Watson, 1982), thaw-mounted on poly-L-lysine-coated slides, and then stored at –70°C until processed. Sections were rapidly brought to room temperature under vacuum, then fixed for 5 min with 4% paraformaldehyde in 0.1*M* sodium phosphate buffer (PBS, pH 7.4). Following 3 × 5 min rinses in PBS, the sections were immersed for 10 min in 0.1*M* triethanolamine containing 0.25% acetic anhydride (pH 8.0). Slides were rinsed in 2X SSC (1X SSC = 0.15*M* NaCl, 0.015*M* sodium citrate [pH 7.0]) then dehydrated 5 min each in 70, 80, and 95% EtOH. Sections were defatted 5 min in chloroform then stored in 95% EtOH at 4°C until used (Wisden et al., 1992).

Hybridization

Sections were prehybridized with 80 μL of hybridization buffer (50% [v/v] formamide, 4X SSC, 1X Denhardt's Reagent, 500 μg/mL heat denatured Herring sperm DNA, 250 μg/mL yeast tRNA, 10% Dextran sulfate) for 2 h under parafilm coverslips in a humidified chamber. A few slides for each oligoprobe were treated with RNase for 30 min (37°C) prior to prehybridization as a negative control. Sections were hybridized at 42°C overnight with 50 μL hybridization buffer plus 10 m*M* DTT and 1×10^6 dpm labeled oligoprobe/section. Coverslips were removed in 2X SSC. Sections were washed to a final stringency of 0.5X SSC at 55°C. The tissue was dehydrated 5 min each in an ascending series of ethanols (70–95%) containing 300 m*M* ammonium acetate (pH 5.5). After 5 min in 100% EtOH, sections were dried in a vacuum desiccator.

Autoradiography and Grain Counting

Slides were dipped in NTB2 emulsion diluted 1:1 with dH_2O and exposed for 10–28 d at 4°C prior to development. The emulsion was developed 2 min in D19 (Kodak), development was stopped 1 min in dH_2O, and was fixed (Kodak Fixer) 2 min. Emulsion-coated sections were then washed in dH_2O and air dried. The sections were lightly stained 5 min in cresyl fast violet (Fluka) and viewed under brightfield or darkfield illumination. Microscopic images over hippocampal (CA1, CA3, CA4, dentate gyrus [DG]) and cortical (II–III, IV, V, and VI) regions of interest were captured with a DAGE camera mounted on a Nikon microscope with the aid of NIH Image software (v.1.41) on a MAC Quadra computer. Grains were counted manually over brightfield images (500X) to obtain a measure of absolute grain density or over darkfield images (125X) using the density threshold option of the image software to obtain a measure of relative grain density. Relative grain density was defined as the "pixel area" covered by grains as a fraction of total pixel area of the brain region of interest. Using the density threshold all grains could be detected independent of relative intensity and therefore measurements were not affected by cell density or staining. Brightfield images were used as a guide for all darkfield measurements. Background was averaged from 2–5 samples ventral to the CA1 pyramidal cell layer over the stratum radiatum which, typical of white matter areas, showed no evidence of specific hybrid-

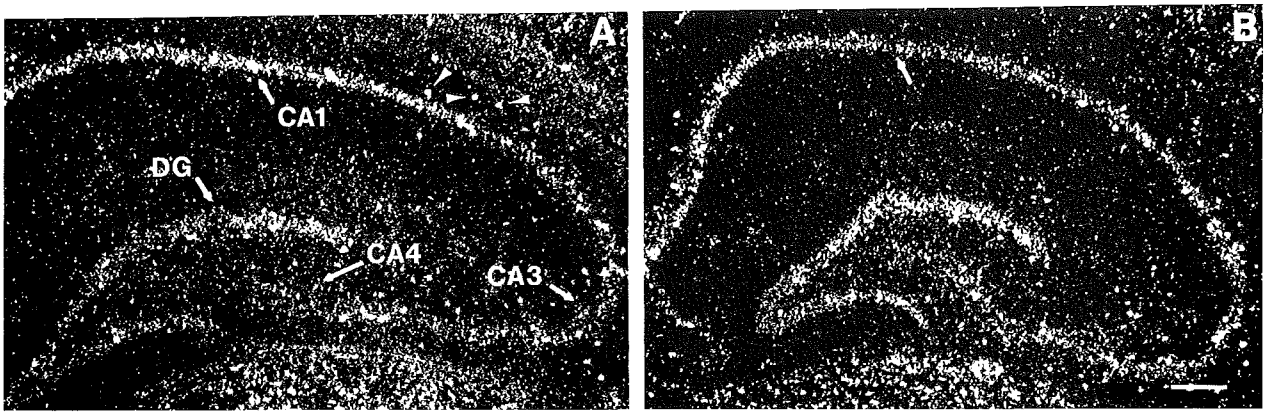

Fig. 1. Representative darkfield photomicrographs (50X) of rat brain sections at the level of the dorsal hippocampus hybridized with an antisense [^{35}S]oligoprobe for the α_1 subunit mRNA of the $GABA_A$ receptor. **(A)** control; **(B)** flurazepam-treated. The relative distribution of α_1 mRNA in the pyramidal cell regions was CA1 > CA3 = DG > CA4 (indicated by arrows). The CA1, CA2, and CA3 regions were also surrounded by numerous densely-labeled, presumably nonpyramidal cells. Three such neurons above the CA1 pyramidal cell layer are indicated with arrowheads. In comparison to control sections (A), there was a significant decrease in absolute (Table 1) and relative (Fig. 2) grain densities over the CA1 region in the flurazepam-treated rat hippocampus (B, arrow). Scale bar = 250 μm.

ization. Background was subtracted from the total grain density measurements to obtain a value for "antisense-specific" grain density for each brain region of interest. Differences in grain density over hippocampal and cortical subregions between flurazepam-treated and control groups was analyzed by multivariate analysis of variance followed by means comparisons using orthogonal contrasts.

Results

The relative distribution of the mRNAs for the α_1, α_5, and γ_2 oligoprobes was similar to that described previously (Wisden et al., 1988, 1992; Khrestchatisky et al., 1989; Olsen and Tobin, 1990). The antisense signal was specific to neurons and was not observed over white matter regions. Uniform, low background labeling was found with RNase-treated sections (results not shown) as was previously found with the sense strands of the α_1, α_5, and γ_2 45-mers used (Wisden et al., 1992).

Expression of α_1 Subunit mRNA

Darkfield photomicrographs of α_1 mRNA distribution in hippocampal formation are shown in Fig. 1. The relative distribution of α_1 mRNA in hippocampus was CA1 > CA3 = DG > CA4 (Fig. 2, Table 1). Consistent with the report of Khrestchatisky et al. (1989), the CA1 region of rat hippocampus is also surrounded by scattered, densely-labeled, presumably nonpyramidal cells (Fig. 1), which were not detected using α_5 or γ_2 subunit probes (Figs. 3 and 4). There was a significant decrease in the expression of the α_1 subunit mRNA in the hippocampal CA1 region of rats orally administered flurazepam, but not in other pyramidal cell layers (Fig. 2). The decrease in α_1 mRNA in CA1 neurons was detected using either method of grain analysis, i.e., absolute or relative grain density measurements (Fig. 2, Table 1).

In frontal cortex the relative abundance of α_1 mRNA was lamina IV > II–III > > V = VI (Figs. 2 and 5). The levels of α_1 mRNA signal in cortical layers may be related to the size and distribution of α_1 mRNA-positive cells in these

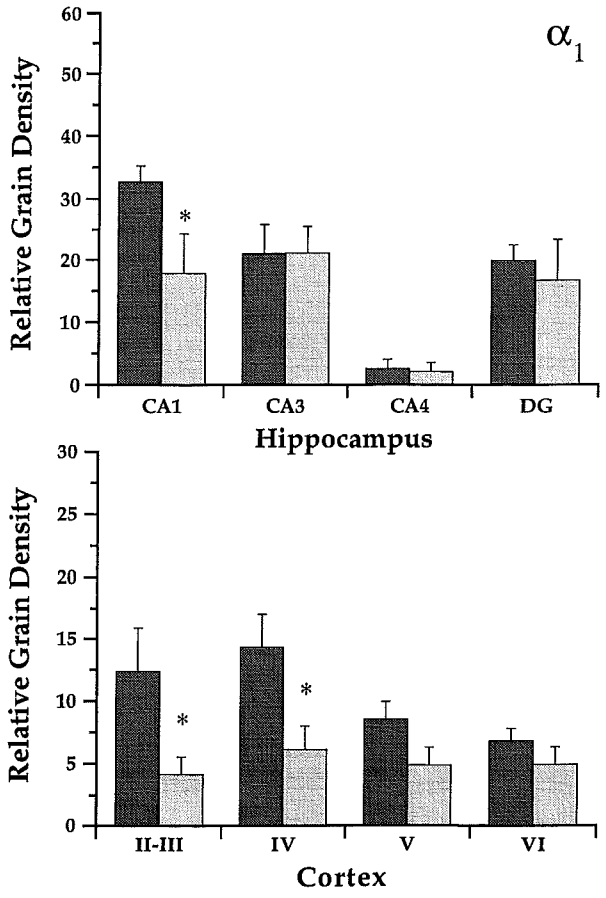

Fig. 2. Relative grain densities (mean ± SEM) corresponding to α_1 mRNA measured in the hippocampus and frontal cortex of control (dark bars) and flurazepam-treated (light bars) rats. There was a significant decrease in α_1 mRNA expression in the CA1 region, but not the other regions, of the hippocampus. Sections hybridized with the α_1 [^{35}S]oligo-probe also showed a decrease in relative grain density in layers II–III and IV of frontal cortex.

layers, i.e., the granule cells in layer IV are more densely packed and labeled whereas the larger pyramidal cells in layer V were densely labeled, but sparsely distributed. Other densely-labeled areas at the coronal levels studied include superior colliculus, medial geniculate, thalamus, and red nucleus. When comparing measures of relative grain density over frontal cortex of chronic flurazepam-treated and control rats, there was a significant decrease in the expression of α_1 mRNA in layers II–III ($p = .05$) and IV ($p = .03$) (Figs. 2 and 5). α_1 mRNA expression in layers V ($p = .06$) and VI ($p = .25$) was not significantly different in comparison to controls. In brightfield images of cortex, cells could not be sampled in sufficient numbers to obtain a reliable measure of absolute grain density across all cell layers. Therefore, only measures of relative grain density are reported.

In other sections cut at the level of the ventral hippocampus (bregma –5.8, Paxinos and Watson, 1982), α_1 subunit mRNA signal was high over substantia nigra pars reticulata (SNpr) neurons as previously reported (Khrestchatisky et al., 1989; Wisden et al., 1992). Of those areas studied, the SNpr had exhibited the largest degree of benzodiazepine receptor downregulation following 1 and 4 wk flurazepam treatment (Tietz et al., 1986). There was no significant difference in absolute grain density over SNpr somata in either ventromedial (control: 1198.2 ± 124.0; flurazepam-treated: 1250.9 ± 179 grains/μm^2, $n = 4$/group, $p = .80$) or dorsolateral (control: 1309.9 ± 61.2; flurazepam-treated: 1257.6 ± 216.8 grains/μm^2, $n = 4$/group, $p = .51$) regions of the SNpr 2 d after the end of treatment (Fig. 6).

Expression of α_5 Subunit mRNA

Similar to the distribution reported by Khrestchatisky et al. (1989) and Wisden et al. (1992), α_5 mRNA was most abundant in the rat hippocampal formation with a relative distribution: CA3 > CA4 = CA1 > > DG (Figs. 3 and 7, Table 1). Caution must be used when making a direct comparison of α_1, α_5, and γ_2 mRNA expression because of differences in oligoprobe labeling efficiency and the exposure time of hybridized sections to the emulsion, which will affect the maximal grain density achieved. However, comparisons of relative subunit distributions can be made within or between regions of interest. For example, α_5 mRNA is particularly abundant in the CA3a–c and CA4 regions in comparison to α_1 and γ_2 mRNAs (Figs. 1 and 4).

Table 1
Absolute Grain Density over Hippocampal Subregions[a]

Subunit mRNA	Hippocampal subregion (grains/1000 μm²)			
	CA1	CA3	CA4	DG
α_1				
Control (n = 6)	50.2 ± 3.5	35.1 ± 4.6	29.4 ± 2.6	24.6 ± 2.8
Treated (n = 6)	34.3 ± 3.9[b]	32.2 ± 2.8	29.7 ± 5.6	23.6 ± 2.8
α_5				
Control (n = 6)	85.6 ± 9.1	107.2 ± 5.9	104.0 ± 4.5	35.7 ± 3.9
Treated (n = 6)	85.5 ± 14.2	82.1 ± 18.5	79.8 ± 16.6	34.2 ± 7.0
γ_2				
Control (n = 5)	111.2 ± 7.5	113.6 ± 13.9	100.8 ± 18.5	119.7 ± 7.6
Treated (n = 6)	115.7 ± 18.3	106.4 ± 17.9	102.8 ± 14.7	123.9 ± 19.4

[a]CA1, CA3, CA4, and dentate gyrus (DG) in control and 1 wk flurazepam-treated rat brain sections following *in situ* hybridization with [^{35}S]-labeled antisense oligoprobes corresponding to the mRNAs for the α_1, α_5, and γ_2 subunit proteins. Values are mean ± SEM.

[b]Denotes significant difference, $p < .01$.

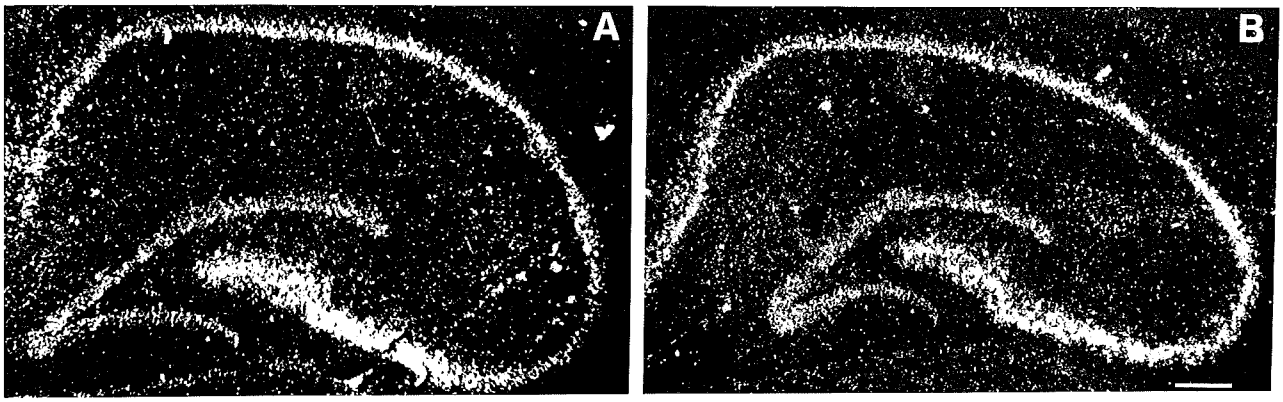

Fig. 3. Representative darkfield photomicrographs (50X) of rat brain sections at the level of the dorsal hippocampus hybridized with an antisense [^{35}S]oligoprobe to the α_5 subunit mRNA for the $GABA_A$ receptor. α_5 mRNA was most abundant in hippocampus with a relative distribution CA3 > CA4 = CA1 > > DG. Note the dense labeling in the CA3 and CA4 areas in comparison to α_1 and γ_2 mRNAs. There were no significant changes in α_5 mRNA expression in hippocampus 2 d after cessation of 1 wk oral flurazepam administration. **(A)** control; **(B)** flurazepam-treated. Scale bar = 250 μm.

In addition, α_5 mRNA was far less abundant in frontal cortex than α_1 or γ_2 mRNAs and did not exhibit a laminar pattern (Fig. 8A) as did α_1 (Fig. 5A) and to some extent γ_2 (Fig. 8B). However, as previously described (Khrestchatisky et al., 1989; Wisden et al., 1992), α_5 mRNA expression showed a laminar distribution within neocortex layer VI (Fig. 8A). Using measures of relative grain density, only CA3 (p = .07) and CA4 (p = .33) regions showed any change in response to chronic flurazepam treatment, although the variability of these responses precluded the detection of significant changes in the hippocampus (see also Table 1; CA3, p = .18 and CA4, p = .15). There were also no significant differences within each CA3 region (a–c) between

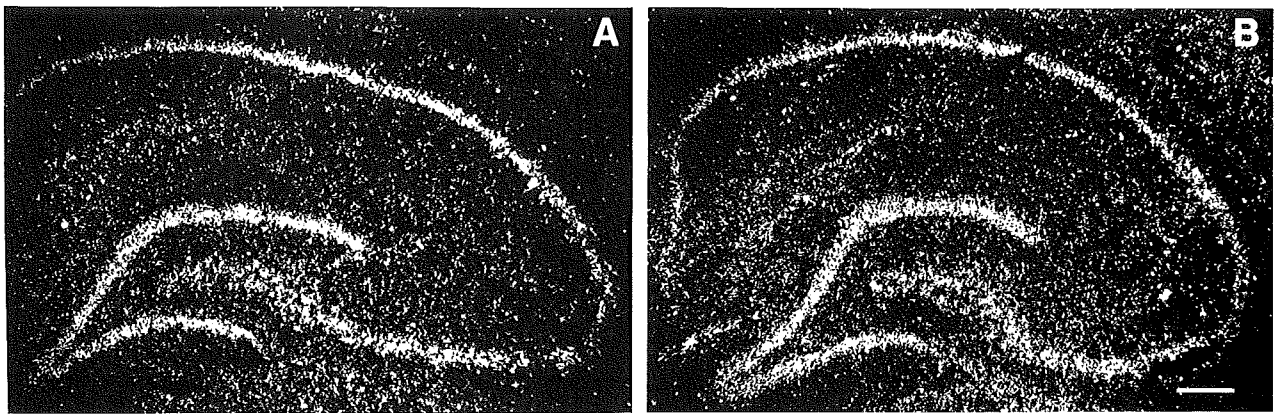

Fig. 4. Representative darkfield photomicrographs (50X) of rat brain sections at the level of the dorsal hippocampus hybridized with an antisense [^{35}S]oligoprobe to the γ_2 subunit mRNA for the $GABA_A$ receptor. γ_2 mRNA was distributed in a pattern similar to α_1 mRNA and, at the coronal level studied, was most abundant in hippocampus (CA1 = CA3 = DG > CA4). There were no significant changes in γ_2 mRNA expression in hippocampus 2 d after cessation of 1 wk oral flurazepam administration. **(A)** control; **(B)** flurazepam-treated. Scale bar = 250 μm.

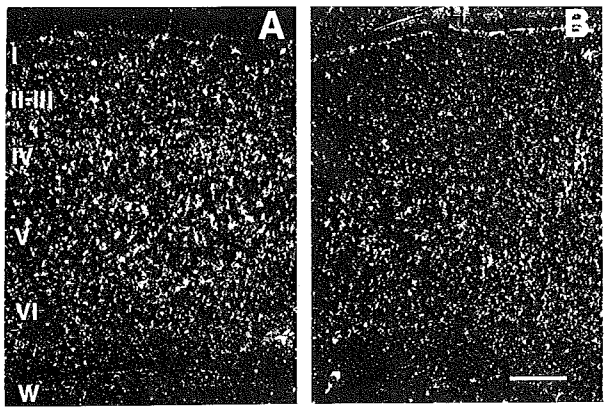

Fig. 5. Representative darkfield photomicrographs (50X) of rat frontal cortex, dorsal to the hippocampal formation, hybridized with an antisense [^{35}S]oligoprobe for the α_1 subunit mRNA of the $GABA_A$ receptor. In neocortex the relative density of mRNA was lamina II–III = IV > V = VI. Following 1 wk oral flurazepam administration, there was a significant decrease in the signal over cortex layers II–III and IV. **(A)** control; **(B)** flurazepam-treated. Scale bar = 250 μm.

groups. As with the hippocampus, there were no significant effects of chronic flurazepam administration on α_5 mRNA expression in any layer of frontal cortex sampled (Fig. 7).

Expression of γ_2 Subunit mRNA

At the coronal level studied, the γ_2 subunit mRNA was most abundant in the hippocampal formation (CA1 = CA3 = DG > CA4) (Fig. 4) and was distributed in a pattern similar to α_1 (Fig. 1) as described by others (Sequier et al., 1988; Khrestchatisky et al., 1989; Shivers et al., 1989). Relative to hippocampus, γ_2 mRNA was less abundant in cortex (Fig. 8). The laminar pattern of expression in cortex was similar to that of the α_1 subunit (Fig. 5), but less prominent. Oral administration of flurazepam did not significantly alter the expression of γ_2 mRNA in any hippocampal region or frontal cortex layer 2 d after the end of treatment (Fig. 9).

Discussion

Using *in situ* hybridization techniques, a discretely localized reduction in $GABA_A$ receptor subunit mRNA expression was found in dorsal hippocampus and frontal cortex of rats 2 d after the end of 1 wk oral flurazepam administration. A decrease in the expression of α_1, but not α_5 or γ_2 mRNA was detected in hippocampal CA1

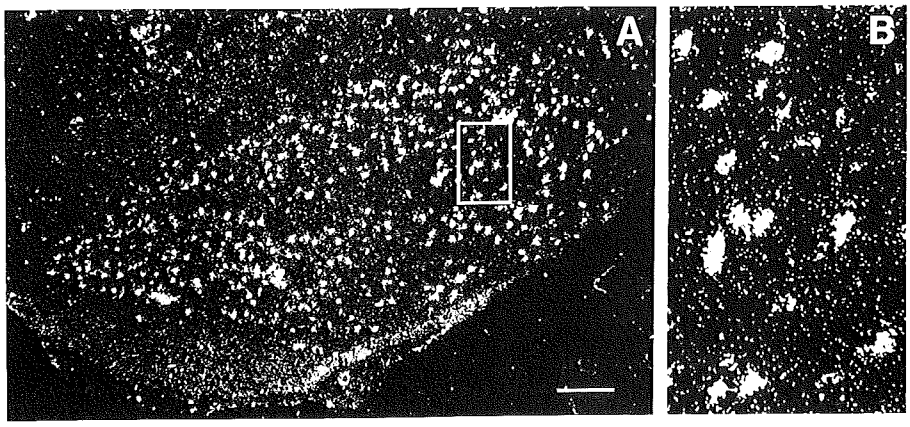

Fig. 6. Representative darkfield photomicrographs of grains over control rat brain at the, level of the caudal substantia nigra pars reticulata (SNpr). Sections were hybridized with an antisense [^{35}S]oligoprobe for the $GABA_A$ receptor α_1 subunit mRNA. **(A)** Entire SNpr (50X) with the majority of neurons prominently labeled. **(B)** Inset in (A) showing grains over individual SNpr cells (125X). There were no differences between treated and control groups in absolute grain density over SNpr neurons. Scale bar = 250 μm.

pyramidal neurons but not in other pyramidal cell layers, nor in the granule cell layer of the dentate gyrus. α_1 mRNA was also selectively reduced in layers II–III and IV of frontal cortex. A similar trend was noted in layer V. The reduced α_1 expression was not a result of nonspecific decreases in mRNA since the changes were confined to specific cell layers and were found in brain regions from the same rats in which no changes in expression of α_5 and γ_2 subunits were observed. The reduction in α_1 subunit expression in several neocortical areas following chronic flurazepam treatment is consistent with earlier reports of a decrease in α_1 expression in cortical homogenates following chronic diazepam (Heninger et al., 1990) or lorazepam (Kang and Miller, 1991) treatments. A decrease in hippocampal α_1 mRNA expression has not previously been reported following prolonged benzodiazepine administration using Northern blot analysis (Heninger et al., 1990; Kang and Miller, 1991; Zhao et al., 1994). *In situ* hybridization seems to be a more sensitive method than Northern blot analysis to detect regional changes in mRNA expression. Given the fraction of the cellular area in which $GABA_A$ receptor subunit mRNAs in hippocampus and cortex are expressed, it may not be unexpected that the discretely localized changes detected in hippocampus in the present study may have previously gone undetected (Heninger et al., 1990; Kang and Miller, 1991; Zhao et al., 1994). The CA1 cell layer comprises <5% of the total hippocampal area and approx 20% of the total pyramidal cell and granule cell layers. In the frontal cortex, on the other hand, in which changes in mRNA expression are more readily detected (Heninger et al., 1990; Kang and Miller, 1991; Primus and Gallager, 1992; Zhao et al., 1994), layers II–III and IV comprise 30–40% of the total cortical area and layer V an additional 25–30% (Bayer and Altman, 1991). The relatively high density of benzodiazepine binding sites in hippocampal CA1 region and layer IV of cortex (Young and Kuhar, 1980; Tietz et al., 1986) may be the reason for the susceptibility of these regions to changes in $GABA_A$ subunit gene expression during chronic benzodiazepine treatment.

A change in $GABA_A$ receptor subunit composition could be related to the reduced sensitivity of hippocampal neurons to GABA and benzodiazepines after chronic benzodiazepine treatment. Oral flurazepam administration

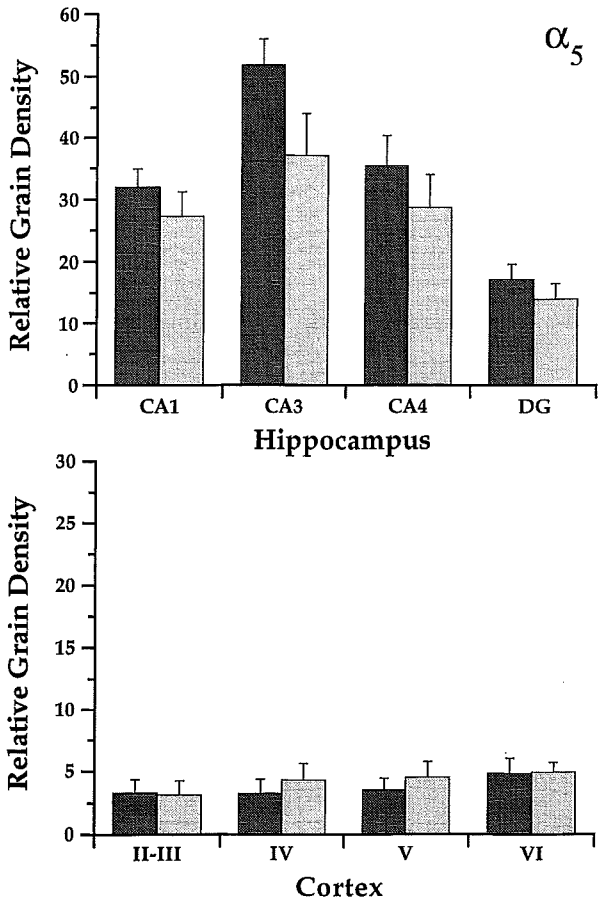

Fig. 7. Relative grain densities over rat brain sections hybridized with the α_5 [^{35}S]oligoprobe. Histograms show mean (± SEM) grain densities measured over the hippocampus and frontal cortex of control (dark bars) and flurazepam-treated (light bars) rats. There were no significant changes in relative grain density, corresponding to α_5 mRNA expression, between fluazepam-treated and control groups in any region of interest.

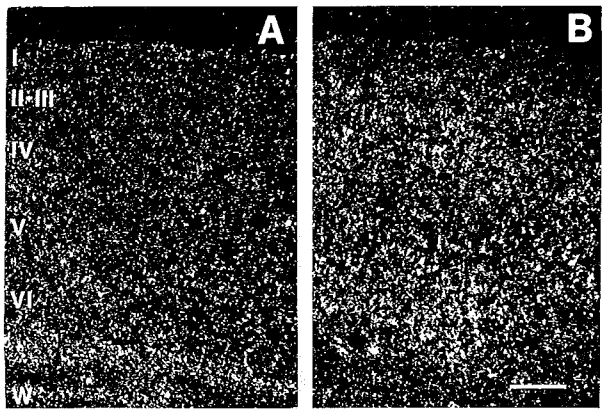

Fig. 8. Representative darkfield photomicrographs (50X) over frontal cortex in control rat brain sections hybridized with an antisense [^{35}S]oligoprobes for the mRNAs of the $GABA_A$ receptor **(A)** α_5 and **(B)** γ_2 subunits. In comparison to α_1 and γ_5 mRNAs, α_5 mRNA was less abundant in cortex and did not show a similar laminar pattern across cortical layers. Note, however, the greater density of labeling in the ventral portion of layer VI. The pattern of expression for γ_2 mRNA was similar to that for α_1. Scale bar = 250 μm.

results in benzodiazepine anticonvulsant tolerance in in vivo (Rosenberg et al., 1991) and a decrease in GABA and benzodiazepine potency, but not efficacy, to inhibit CA1 pyramidal cells in in vitro hippocampus (Xie and Tietz, 1992). A decrease in α_1 subunit mRNA expression in the CA1 region following chronic flurazepam treatment could have resulted in this reduction of $GABA_A$ agonist and diazepam potency (Xie and Tietz, 1992). Conversely, the lack of change in α_1 mRNA expression in the CA3 pyramidal layer is consistent with the lack of change in the sensitivity of CA3 neurons to locally applied GABA following chronic diazepam administration (Lista et al., 1990). Recombinant $GABA_A$ receptors assembled from different combinations of α, β, and γ subunits show different GABA and benzodiazepine sensitivities. Studies have indicated that variation in α and β subunit isoforms alters GABA-induced chloride currents (Levitan et al., 1988; Sigel et al., 1990). The presence of a γ_2 subunit also altered GABA sensitivity and was required for diazepam-induced potentiation of GABA-activated currents (Pritchett et al., 1989a; Sigel et al., 1990; Angelotti and Macdonald, 1993). Despite the importance of the γ_2 subunit for benzodiazepine pharmacology, the lack of change in γ_2 subunit mRNA expression suggests

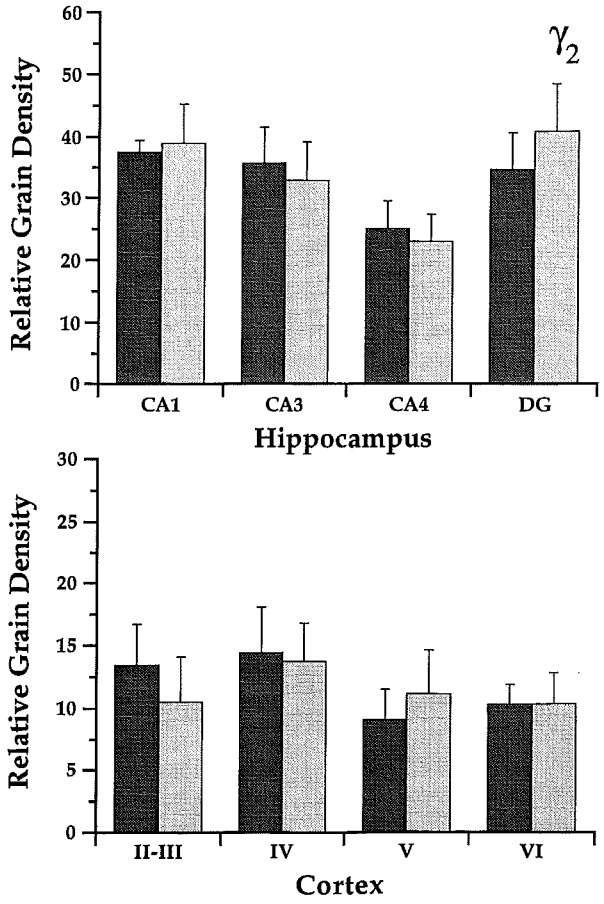

Fig. 9. Relative grain densities over rat brain sections hybridized with the γ_2 [^{35}S]oligoprobe. Histograms show mean (± SEM) grain densities measured over the hippocampus and frontal cortex of control (dark bars) and flurazepam-treated (light bars) rats. There were no significant changes in relative grain density, corresponding to γ_2 mRNA expression, between flurazepam-treated and control groups in any region of interest.

that the decrease in diazepam potency measured in in vitro hippocampal slices 2 d after the end of flurazepam administration (Xie and Tietz, 1992) may be the result of changes in the α_1 or other $GABA_A$ subunits but not the γ_2 subunit.

The relationship between $GABA_A$ receptor mRNA expression and changes in numbers of binding sites at the GABA receptor following various chronic treatment regimens is unresolved. Montpied et al. (1991) reported a close temporal correlation between reductions in [^{3}H]flunitrazepam binding and decreased α_1 and α_2 subunit mRNA expression in embryonic chick cultures following chronic GABA treatment. One and 4 wk chronic flurazepam treatment decreased [^{3}H]flunitrazepam binding in hippocampus and layer IV of cortex as well as other brain regions, including SNpr, although this downregulation had reversed by 2 d after 4 wk treatment (Tietz et al., 1986). Preliminary autoradiographic studies indicated no change in muscimol binding sites in stratum pyramidale or stratum radiatum of hippocampus after 4 wk of flurazepam administration (Tietz, unpublished data). Since [^{3}H]flunitrazepam binding sites in hippocampus were likely to have returned to pretreatment levels in rat brains examined in the present study (Tietz et al., 1986), the reduction in α_1 subunit mRNA expression may not be temporally correlated with decreased benzodiazepine receptor density. The lack of correlation between α_1 mRNA expression and changes in benzodiazepine receptor density is, however, a consistent finding following chronic benzodiazepine treatment. For example, although continuous lorazepam administration for 1 wk decreased [^{3}H]flunitrazepam binding in cortex and hippocampus of mice, α_1 mRNA expression in cortex was only decreased after 2 wk of continuous drug administration and no change was detected in hippocampus (Kang and Miller, 1991). As suggested by the authors, the inability to detect a change in mRNA expression in hippocampus may have been because of the lesser sensitivity of the method used (Kang and Miller, 1991). Subcutaneous diazepam treatment for 3 wk has not been reported to induce benzodiazepine receptor downregulation (Gallager et al., 1991; Wu et al., 1994), but did result in decreased α_1 subunit expression in cortex (Heninger et al., 1990). γ_2 subunit mRNA expression was also reduced in cortex after more prolonged benzo-

diazepine treatments (Kang and Miller, 1991; Primus and Gallager, 1992) including 4 wk oral flurazepam treatment (Zhao et al., 1994). In the latter study, in which the γ_2 subunit mRNA was reduced in the hippocampus after the more prolonged flurazepam administration, the time-course for reduction and recovery of γ_2 mRNA levels correlated with downregulation of benzodiazepine binding in brain homogenates. Whether discretely localized changes in the expression of α_1 or γ_2 mRNA occur immediately after discontinuation of 1 wk flurazepam treatment, when benzodiazepine receptors are down-regulated, remains to be studied.

α_5 subunit mRNA expression was investigated for several reasons. The α_5 transcript is expressed primarily in the hippocampus and in the cortex and olfactory bulb (Wisden et al., 1988, 1992; Malherbe et al., 1990). In addition, the α_5 subunit was shown in solution hybridization experiments of whole rat brain to be decreased after 2 h to 16 d of flurazepam exposure (40 mg/kg/d, ip) (O'Donovan et al., 1992a). With continued flurazepam injections, α_3 and α_6 subunit mRNA expression were reported to increase whereas expression of the mRNA for all other α and $\beta_{(1-3)}$ variants tested, as well as for the γ_2 subunit, did not change (O'Donovan et al., 1992a,b). These findings are of particular interest since they showed that increased, as well as decreased, expression of $GABA_A$ receptor subunit mRNAs can occur during chronic flurazepam administration. This could result in changes in $GABA_A$ receptor subunit composition, possibly allowing for changes in $GABA_A$ receptor pharmacology even in the absence of a decrease in benzodiazepine binding (Pritchett et al., 1989b; Pritchett and Seeburg, 1990, 1991; Sigel et al., 1990). Although there was a trend toward a decrease in the CA3 and CA4 regions, α_5 subunit mRNA expression did not significantly change in hippocampus or cortex following 1 wk of oral flurazepam administration, a treatment that is different from that of O'Donovan et al. (1992a,b). Using oral flurazepam treatment as in the present study, Zhao et al. (1994) recently reported a decrease in α_5 mRNA immediately after the end of 2 wk, but not after 4 wk, of flurazepam treatment, and a significant decrease in hippocampus 4 h after a single ip injection of flurazepam. Thus, the α_5 subunit mRNA appears to be capable of rapid change, and a decrease may no longer be robust 2 d following cessation of treatment.

The presence of an α_1 subunit in the $GABA_A$ receptor has been associated with binding of ligands that are selective for the Type I (BZ1) benzodiazepine receptors (Young et al., 1981; Pritchett et al., 1989b; Olsen and Tobin, 1990; Pritchett and Seeburg, 1990) although the correlation between α_1 transcripts and Type I binding is not absolute (Bateson et al., 1991). The decrease in α_1 mRNA might suggest a selective loss of BZ1 sites in flurazepam-treated rats. Indeed, the binding of the BZ1-selective ligand, [^{3}H]zolpidem (Wu et al., 1994), like [^{3}H]flunitrazepam binding (Tietz et al., 1986; Wu et al., 1994), is decreased in hippocampus and cortex of rats administered oral flurazepam for 4 wk. Of those regions evaluated in the present study, cortex layer IV and SNpr contain predominantly Type I sites. Nontype I (Type II) sites are enriched in cortex layer I–III, the CA1 region of hippocampus, and the dentate gyrus (Young et al., 1981; Olsen et al., 1990). No consistent pattern of decreased mRNA expression related to the distribution of benzodiazepine receptor subtypes emerged from the study of α_1 and α_5 subunits 2 d after the end of drug administration.

Based on the literature, it is possible to propose a regional localization of $GABA_A$ receptor subtypes on pyramidal neurons. Thus, changes in receptor subunit composition might produce particular changes in the functional organization of the GABA inhibitory system in the hippocampus, and may be the basis for our results in the in vitro hippocampal slice (Xie and Tietz, 1991, 1992; Zeng et al., 1993, 1994). Hippocampal GABA inhibition in the CA1 region is mediated by several classes of GABAergic interneurons that synapse on the pyramidal cell soma (recurrent

and feedforward inhibition) and dendrites (feedforward inhibition) (Lacaille et al., 1989). $GABA_A$ receptor subunit proteins have been identified immunohistochemically to be differentially distributed throughout hippocampal lamina corresponding to the different anatomical components of the pyramidal cell. For example, the α_1, γ_2, and $\beta_{2/3}$ subunit proteins have been localized to the apical and basal dendritic layers of pyramidal cells (Benke et al., 1991; Fritschy et al., 1992). The less abundant α_3 transcript was localized to somal and apical dendritic layers (Fritschy et al., 1992). Consistent with the heterogeneous distribution of subunit proteins, subpopulations of $GABA_A$, receptors have been detected in both somal and dendritic layers of hippocampus (Young and Kuhar, 1980; Tietz et al., 1986; Faull and Villiger, 1988; Sequier et al., 1988; Olsen et al., 1990). Further, based on electrophysiological and pharmacological evidence, two distinct populations of dendritic $GABA_A$ receptors, i.e., synaptic and extrasynaptic, have been proposed to mediate GABA-activated inhibition in CA1 pyramidal cell dendrites (Alger and Nicoll, 1982). GABA induces hyperpolarization in both the soma and the dendrites, and an inhibitory depolarization only in dendrites. Although both responses are sensitive to blockade by $GABA_A$ antagonists, the depolarizing response is more sensitive to bicuculline (Alger and Nicoll, 1982; Wong and Watkins, 1982). Further, the $GABA_A$ agonist, THIP, which binds to a subset of GABA receptors (Falch and Krogsgaard-Larsen, 1982), preferentially induced dendritic hyperpolarization (Alger and Nicoll, 1982). The depolarizing IPSP is also less sensitive than the hyperpolarizing IPSP to diazepam (Alger and Nicoll, 1982). This latter finding could be interpreted as evidence for benzodiazepine-coupled and non-benzodiazepine-coupled $GABA_A$ receptors on CA1 pyramidal cell dendrites. Thus, $GABA_A$ receptor heterogeneity caused by differential assembly of subunit proteins on the soma and dendrites of CA1 pyramidal neurons may be responsible for their characteristic biphasic response to GABA and the differential sensitivity of these responses to benzodiazepines.

The above findings suggest how changes in subunit composition could relate to a decrease in GABA function in benzodiazepine-tolerant rat hippocampus. The dysfunction of the GABA inhibitory system in the hippocampus of benzodiazepine-tolerant rats may be attributable to a selective action on benzodiazepine-coupled $GABA_A$ receptors in the CA1 region that could lead to altered gene expression of specific $GABA_A$ receptor subunit genes. Since the α_1 subunit protein was localized to dendrites of pyramidal cells, it would imply that at least dendritic GABA-mediated inhibition may be reduced following chronic flurazepam treatment. Indeed, recurrent (Xie and Tietz, 1991) and feedforward (Zeng et al., 1994) inhibition were found to be reduced in the CA1 region following 1 wk of oral flurazepam administration. In addition, it has been demonstrated that the reduction in the fast, hyperpolarizing PSP in CA1 pyramidal cells of benzodiazepine-tolerant rats (Zeng et al., 1993) unmasks a typical depolarizing IPSP (Zeng et. al., unpublished data), perhaps mediated by nonbenzodiazepine-coupled GABA receptors. If changes in $GABA_A$ subunit mRNA expression are central to decreased GABA inhibition in hippocampus, then the fact that recurrent, primarily somatic (Lacaille et al., 1989) inhibition was also reduced in flurazepam-treated rats (Xie and Tietz, 1991) would suggest that additional $GABA_A$ subunits associated with functionally distinct $GABA_A$ receptors in pyramidal cell soma may also be regulated in hippocampus following chronic flurazepam treatment.

The major finding of this report was a decreased expression of the mRNA encoding the α_1 subunit of the $GABA_A$ receptor in the rat hippocampus and cortex 2 d after the end of oral flurazepam administration. The decrease in α_1 mRNA expression in the CA1 region of the hippocampus is consistent with the decreased sensitivity to GABA in benzodiazepine-tolerant rat hippocampus and may be related to the

decrease in GABA-mediated inhibition in this region (Xie and Tietz, 1991; Zeng et al., 1993, 1994). Caution must be used in interpreting relative levels of mRNA expression in relation to changes in receptor number and function since it is not known whether the changes in mRNA are reflected in alterations in functional receptors. A systematic study of the regulation and time-course of changes in $GABA_A$ subunit (α_{1-5}, β_{1-3}, and γ_2) expression and in the number and affinity of GABA, benzodiazepine, and other allosteric binding sites in the CA1 region in conjunction with a quantitative immunohistochemical evaluation of subunit proteins will be necessary to address these questions directly. The discrete nature of the functional and molecular changes that have been detected in the hippocampus of benzodiazepine-tolerant rats suggests that the study of the electrophysiological function and cellular and molecular pharmacology in the CA1 region of chronic flurazepam-treated rats provides a valuable model for the study of the synaptic mechanisms of benzodiazepine tolerance.

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