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Rapid down-regulation of [^{3}H]zolpidem binding to rat brain benzodiazepine receptors during flurazepam treatment

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Abstract

In a previous study, it was found that down-regulation of benzodiazepine (BZ) binding in rats treated 4 weeks with flurazepam was relatively greater and more widespread when measured with [^{3}H]zolpidem, a selective 'BZ_1 receptor' ligand, than that measured with the non-selective ligand, [^{3}H]flunitrazepam. In the present study, the time course for down-regulation of [^{3}H]zolpidem binding was studied in rats treated with flurazepam. [^{3}H]Zolpidem binding was also studied in rats given a midazolam treatment shown to cause tolerance. Rats were chronically treated with flurazepam for 1 or 2 weeks, or with midazolam for 3 weeks, then killed immediately after the treatment. Another group of rats was acutely treated with desalkyl-flurazepam and killed 30 min later. After 2 weeks of flurazepam treatment, the B_{max} of [^{3}H]zolpidem binding was decreased by 22% in cerebral cortex, 26% in cerebellum and 33% in hippocampus, with no change in the K_d in any region. After 1 week of flurazepam treatment, the B_{max} was decreased by 23% in cerebellum and 14% in hippocampus, but not changed in cerebral cortex. The K_d was increased in cerebral cortex, but not in cerebellum or hippocampus. Neither the B_{max} nor the K_d of [^{3}H]zolpidem binding was affected by acute desalkyl-flurazepam treatment, or by 3 weeks of midazolam treatment. These results, in combination with previous findings, which showed no change in [^{3}H]flunitrazepam binding after 1 or 2 week flurazepam treatment, and no change in cerebellum even after the 4 week treatment, may indicate a shift in BZ receptor subtypes in flurazepam-tolerant rats. Such a shift could be based on possible changes in the subunit composition or conformation of $GABA_A$/BZ receptors after 1 or 2 week flurazepam treatment.

Keywords: Benzodiazepine type I receptor; [^{3}H]Zolpidem; Tolerance; Receptor down-regulation; Midazolam; Flurazepam

1. Introduction

Benzodiazepines (BZs) act via BZ receptors, which are located within $GABA_A$ receptors. Biochemical and pharmacological studies indicated the existence of two distinct BZ receptor subtypes (BZ_1 and BZ_2 receptors) (Braestrup and Nielsen, 1981; Braestrup et al., 1982; Klepner et al., 1979). The BZ_1 receptor has high affinity for β-carbolines, CL 218,872 (3-methyl-6-[3-trifluoromethyl-phenyl]-1,2,4-triazolo[4,3-*b*]pyridazine) and zolpidem, while the BZ_2 receptor has low affinity for these compounds (Braestrup and Nielsen, 1981; Braestrup et al., 1982; Klepner et al., 1979). It was found that the particular α subunit isoform included in the $GABA_A$ receptor defines the BZ receptor subtypes, e.g., the α_1 subunit bestows BZ_1 receptor characteristics, while the presence of α_2, α_3 or α_5 subunits determines BZ_2 receptor properties (Pritchett et al., 1989; Pritchett and Seeburg, 1990). However, recent findings have suggested that the actual BZ receptor subtypes are more complicated than a simple classification as BZ_1 and BZ_2 receptors. For example, expressing different α subunits in combination with β_3 and γ_2 subunits showed that zolpidem did not bind to receptors containing the α_5 subunit, bound with relatively low affinity to receptors containing α_2 or α_3 subunits, and bound with high affinity to receptors containing α_1 subunits (Pritchett and Seeburg, 1990). An even more complicated picture emerged when binding was studied to rat brain receptors immunoprecipitated with subunit-specific antibodies (Mertens et al., 1993). Though zolpidem bound with highest affinity to receptors precipitated with the anti-α_1 antibody, it also bound to receptors precipitated with the anti-α_3 or anti-α_5 antibodies. The α_5 subunit was found to be associated with

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α_1 and α_3 subunits in these native receptors (Mertens et al., 1993). In the same study, flunitrazepam bound with similar high affinity to receptors precipitated with antibodies directed against α_1, α_3 or α_5 subunits (Mertens et al., 1993). The term 'BZ_1 receptors' in the present work is used to refer to those receptors detected by high affinity zolpidem binding, though this may not constitute a single, homogeneous population of receptors.

Various lines of evidence have shown that chronic benzodiazepine administration results in the development of functional tolerance (Rosenberg and Chiu, 1985; Rosenberg et al., 1985), and many neurochemical changes that may be related to tolerance have been reported. Four week flurazepam treatment of rats decreased the [^{3}H]flunitrazepam binding to BZ receptors in cerebral cortex and hippocampus, caused tolerance to flurazepam-induced motor impairment (Rosenberg and Chiu, 1981a), and decreased the ability of benzodiazepines to potentiate GABA-stimulated Cl^- influx (Yu et al., 1988). Tolerance after shorter flurazepam treatments has also been found. Rats that were treated with flurazepam for only 1 week were tolerant to the anticonvulsant effect of several benzodiazepines (Rosenberg et al., 1991) and the rotational behavior in response to infusion of flurazepam into substantia nigra pars reticulata (Tietz and Rosenberg, 1988). After 1 week flurazepam treatment, the inhibitory effect of flurazepam on the spontaneous activity of neurons in the substantia nigra pars reticulata was decreased (Tyma et al., 1988), as were the potency of $GABA_A$ receptor agonists and of diazepam in the in vitro hippocampal slice (Xie and Tietz, 1992). This treatment also decreased the potentiation by benzodiazepines of the GABA-stimulated Cl^- influx, although it did not change the GABA-stimulated Cl^- influx itself (Li et al., 1993; Ngur et al., 1990). BZ receptor binding has also been studied, although the relationship between down-regulation of the BZ receptor and tolerance remains unclear. Using brain homogenate binding assays, no change in [^{3}H]flunitrazepam binding was found in cerebral cortex after 1 or 2 week flurazepam treatment (Rosenberg and Chiu, 1981a), although an autoradiographic study showed that there were localized decreases in [^{3}H]flunitrazepam binding in some regions, including cortical layer IV and hippocampal dentate gyrus, after a week of treatment (Tietz et al., 1986).

Recently, we compared the binding of [^{3}H]zolpidem, a selective BZ_1 receptor agonist (Arbilla et al., 1985, Arbilla et al., 1986), to that of [^{3}H]flunitrazepam, a non-selective benzodiazepine agonist. It was found that 4 week flurazepam treatment produced a relatively greater reduction in BZ_1 receptors in cerebral cortex and hippocampus than was measured for the total BZ receptor population (Wu et al., 1994). In that study, the greatest difference between [^{3}H]zolpidem binding and [^{3}H]flunitrazepam binding was found in cerebellum, in which the B_{max} of [^{3}H]flunitrazepam binding was not changed (Rosenberg and Chiu, 1981b; Wu et al., 1994), but a 32% decrease in the BZ_1 receptor was found (Wu et al., 1994). These results suggested that the down-regulation of BZ receptors after chronic flurazepam treatment primarily involves the BZ_1 receptor. Since over 90% of cerebellar BZ receptor binding in controls is of the BZ_1 subtype, and as there was no change in total cerebellar BZ receptor binding (i.e. [^{3}H]flunitrazepam binding), the previous results also implied that the number of BZ receptors that are labelled by [^{3}H]flunitrazepam but not by [^{3}H]zolpidem must have been increased. A similar conclusion was drawn from the results found after chronic lorazepam treatment (Galpern et al., 1990). Based on these results, it was hypothesized that shorter flurazepam treatments, which had not altered the binding of the non-selective ligand, [^{3}H]flunitrazepam, might affect the binding of the BZ_1-selective ligand, [^{3}H]zolpidem. Thus, in the present work, the effect of chronic flurazepam treatment for 1 and 2 weeks on [^{3}H]zolpidem binding was studied. The present study also evaluated the effects of chronic midazolam treatment on [^{3}H]zolpidem binding. Previous studies showed that chronic exposure to midazolam also produced tolerance to its anticonvulsant action, although no change in [^{3}H]flunitrazepam binding was found in cerebral cortex, hippocampus or cerebellum (Ramsey-Williams et al., 1994).

2. Materials and methods

2.1. Animals

Experiments were performed using male Sprague-Dawley rats, with initial weight of 175–199 g for 1 and 2 week flurazepam treatment, and 150–174 g for 3 week midazolam treatment. When used for binding assay at the end of chronic treatment, rats weighed 250–300 g. The weight of rats for acute treatment was 250–300 g. Animals were housed in a room with a 12 h light/dark cycle (light on at 6 a.m., off at 6 p.m.). Rats were chronically treated with flurazepam for 1 or 2 weeks, or midazolam for 3 weeks, or acutely treated with desalkyl-flurazepam. Each control group was given drug vehicle and was handled identically for the same time period as their matched treated group.

2.2. Chronic flurazepam treatment

Flurazepam in 0.02% saccharin solution was provided as drinking water for 1 or 2 weeks using a standard protocol (Rosenberg and Chiu, 1981a). The

flurazepam concentration was adjusted based on the weight and fluid consumption of the rat, so that it was allowed access to 100 mg/kg for the first 3 days and 150 mg/kg for the remainder of the treatment for 1 week treated rats, or 100 mg/kg for the first week and 150 mg/kg for the second week for 2 week treated rats. Controls were given 0.02% saccharin solution and handled identically. Rats were killed immediately after the end of chronic treatment.

2.3. Acute desalkyl-flurazepam treatment

Rats were acutely treated with desalkyl-flurazepam according to a previous study (Xie and Tietz, 1992). Briefly, 2.5 mg/ml desalkyl-flurazepam was dissolved in an emulsion of peanut oil, water and acacia (4:2:1). The food was removed from the cage 12 hours before intubation. A dose of 2.5 mg/kg of desalkyl-flurazepam was given to rats by gastric intubation. The control rats were given emulsion only. Rats were killed 30 min later. A previous study showed that this treatment resulted in a level of brain benzodiazepine activity equivalent to that of 1 week chronic flurazepam treatment (Xie and Tietz, 1992).

2.4. Chronic midazolam treatment

Midazolam was administered as in a previous study (Ramsey-Williams et al., 1994). Rats were given as their sole source of fluid either 0.02% saccharin solution (control) or saccharin solution containing midazolam. A 20 mg/ml stock solution of midazolam, dissolved in 0.1 N HCl, was used to prepare the midazolam-containing saccharin solution. The maximal midazolam concentration was 0.5 mg/ml. Based on each rat's body weight and volume of fluid consumed, the midazolam concentrations were adjusted so that rats received a daily dose of 40 mg/kg. Rats were killed immediately after the 3 week treatment.

2.5. Brain membrane preparation

Immediately after rats were killed by decapitation, brains were quickly removed, placed on ice, and dissected. Three brain regions (cerebral cortex, cerebellum and hippocampus) were removed and frozen at −70° C until the day of the assay. The tissue was homogenized in 0.32 M sucrose in a glass homogenizer using a Teflon pestle. The homogenates were centrifuged at $1000 \times g$ for 10 min at 4° C. The supernatant was re-centrifuged at $20000 \times g$ for 20 min. The resulting pellet (P_2) was re-suspended in 50 mM Tris-HCl buffer (pH 7.4 at 0° C) and centrifuged again. This was repeated one more time to prepare the 'triple-washed' synaptosomal fraction as previously described (Chiu and Rosenberg, 1978; Wu et al., 1994). This procedure was expected to remove most of the residual flurazepam and metabolites which might remain in the brain following chronic treatment, and which could interfere with the binding assay, as shown in the previous study (Wu et al., 1994). Most of the endogenous GABA will also be removed by this procedure (Chiu and Rosenberg, 1979), although some 'tightly bound' GABA will remain (Napias et al., 1980). The pellet was finally re-suspended in 50 mM Tris-HCl buffer. Tissue from two rats was pooled for binding assay of the hippocampus.

2.6. [^{3}H]Zolpidem binding assay

Binding assays were performed as previously described (Wu et al., 1994). [^{3}H]Zolpidem (1–40 nM) was used as the radioligand, and the reaction volume was 0.5 ml. The protein concentration in the reaction mixture was 0.15–0.5 mg/ml. Reaction tubes were incubated at 0° C for 60 min. Non-specific binding of [^{3}H]zolpidem was determined by including 40 μM clonazepam in the incubation mixture. Specific binding was defined as the difference between the total binding and non-specific binding. At the end of the incubation, the reaction was stopped by adding 5 ml of ice-cold 50 mM Tris-HCl buffer and rapid filtration through Whatman GF/B glass fiber filters, followed by washing two more times with 5 ml ice-cold 50 mM Tris-HCl buffer. The filters had been pre-soaked in 0.3% polyethylenimine in Tris-HCl buffer overnight. Under these conditions, the specific binding was over 80% of total binding for [^{3}H]zolpidem at 1 nM. All filters were placed in glass scintillation vials with 5 ml Cytoscint scintillation cocktail (ICN Biomedicals, Irvine, CA) overnight, and then counted using a Beckman scintillation counter. The counting efficiency was about 48%. Protein concentration was determined using the bicinchoninic acid reagent kit (Pierce Biochemicals, Rockford, IL) using bovine serum album as the reference standard.

2.7. Data analysis

Linear regression analysis of Scatchard plots was used to determine the maximal binding capacity (B_{max}) and dissociation constant (K_d). Student's t-test was used to determine the significance of difference between treated and control groups for both B_{max} and K_d. In all cases, $P < 0.05$ was considered significant.

3. Results

3.1. [^{3}H]Zolpidem binding after 1 or 2 week flurazepam treatment

Scatchard analysis of [^{3}H]zolpidem binding to brain membranes from flurazepam-treated rats showed that,

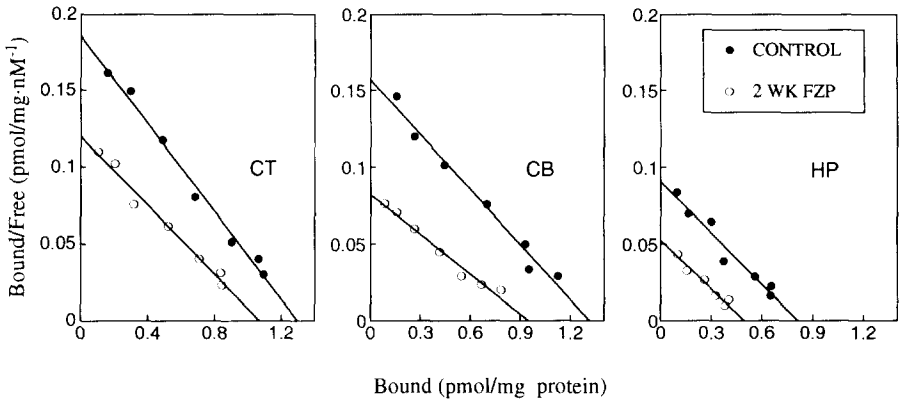

Fig. 1. Representative Scatchard plots of the specific binding of [^{3}H]zolpidem to brain membranes from the cerebral cortex (CT), cerebellum (CB) and hippocampus (HP) of control rats and rats studied immediately after 2 weeks of flurazepam treatment.

as in controls, zolpidem bound to a single population of recognition sites (Fig. 1). Compared to binding in controls, the B_{max} of [^{3}H]zolpidem binding in brain membranes from 2 week flurazepam treated rats was significantly reduced in cerebral cortex, hippocampus and cerebellum (Figs. 1 and 2). In cerebral cortex, the B_{max} of [^{3}H]zolpidem binding was reduced by 22% ($P < 0.05$); in cerebellum, by 26% ($P < 0.01$); and in hippocampus, by 33% ($P < 0.05$). In all three brain regions, the dissociation constant (K_d) of [^{3}H]zolpidem tended to be greater in flurazepam-treated rat brain membranes, but this was not statistically significant (Fig. 2). These results were similar to those after 4 week flurazepam treatment (Wu et al., 1994).

Similar to the effects of 2 week flurazepam treatment, 1 week flurazepam treatment reduced the B_{max} of [^{3}H]zolpidem binding in cerebellum (22.7%) and hippocampus (14.5%), but not in cerebral cortex. The K_d in cerebral cortex was increased significantly, but not in the other two regions (Fig. 3).

3.2. [^{3}H]Zolpidem binding after acute desalkyl-flurazepam treatment

Previous studies showed that gastric intubation of rats with 2.5 mg/kg desalkyl-flurazepam can produce a brain level of desalkyl-flurazepam similar to that after the chronic flurazepam treatment used in the present study (Xie and Tietz, 1992). Using this acute treatment, rats did not show any obvious ataxia or sedation after treatment. It should also be noted that rats consuming flurazepam in the drinking water did not show sedation or obvious motor impairment (Rosenberg and Chiu, 1981a). However, in contrast to the effect of chronic flurazepam treatment, acute desalkyl-flurazepam treatment had no significant effect on the B_{max} or the K_d of [^{3}H]zolpidem binding in the cerebellum or hippocampus (Fig. 4). This result, combined with the results in cerebral cortex after 1 week flurazepam treatment, which showed no difference in [^{3}H]zolpidem binding compared to the control, indicated that the decreases in the B_{max} of [^{3}H]zolpidem binding were specific to chronic treatment.

3.3. [^{3}H]Zolpidem binding after 3 week midazolam treatment

Chronic treatment of rats with midazolam for 3 weeks did not change the B_{max} or K_d of [^{3}H]zolpidem binding in cerebral cortex, hippocampus or cerebellum

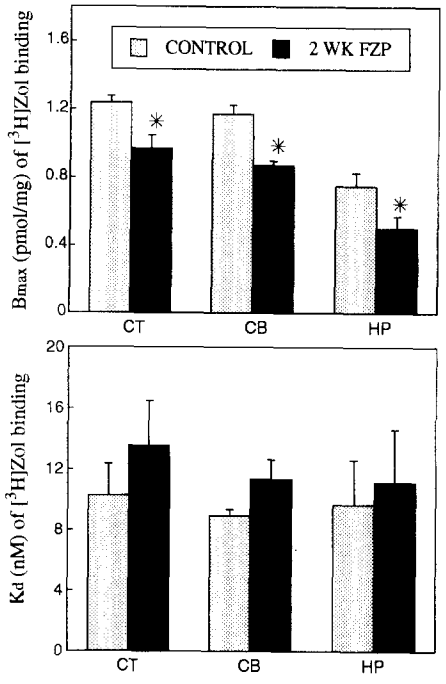

Fig. 2. Specific binding of [^{3}H]zolpidem to cerebral cortex (CT), cerebellum (CB) and hippocampus (HP) of control rats, and rats sacrificed immediately after 2 week flurazepam treatment. The bars represent the mean + S.E.M. of four independent experiments (* $P < 0.05$ compared to control). The B_{max} was significantly decreased in all three areas immediately after the 2 week flurazepam treatment. The K_d of [^{3}H]zolpidem binding was not significantly changed after chronic flurazepam treatment.

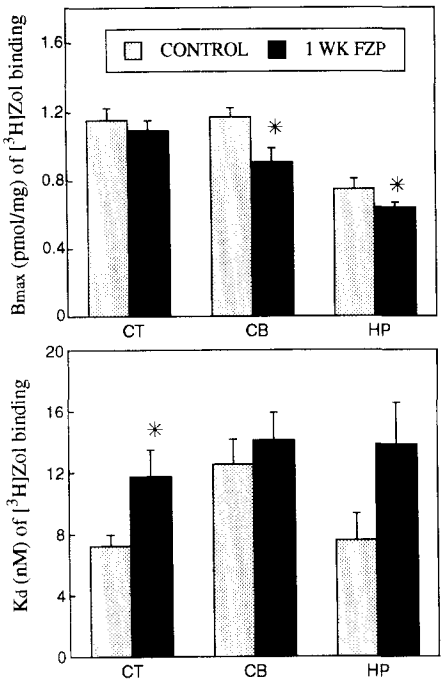

Fig. 3. Specific binding of [^{3}H]zolpidem to cerebral cortex (CT), cerebellum (CB) and hippocampus (HP) of control rats, and rats sacrificed immediately after completing 1 week flurazepam treatment. The bars represent the mean + S.E.M. of four to eight independent experiments (* $P < 0.05$ compared to control). The B_{max} was significantly decreased in cerebellum and hippocampus, but not in cerebral cortex. The K_d in cerebral cortex was significantly increased after 1 week flurazepam treatment.

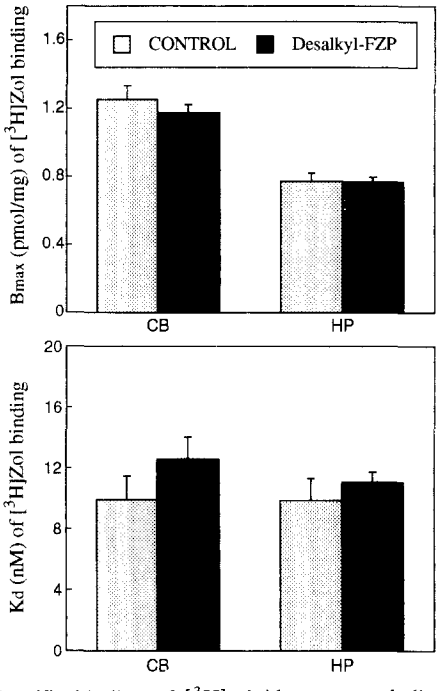

Fig. 4. Specific binding of [^{3}H]zolpidem to cerebellum (CB) and hippocampus (HP) of control rats, and rats killed 30 min after acute gastric intubation with 2.5 mg/kg desalkyl-flurazepam. The bars represent the mean + S.E.M. of three or four independent experiments. Acute desalkyl-flurazepam treatment had no significant effect on the B_{max} or K_d of [^{3}H]zolpidem binding.

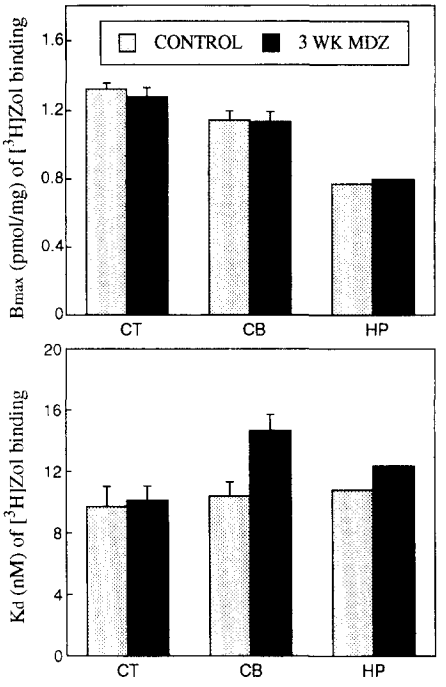

Fig. 5. Specific binding of [^{3}H]zolpidem to cerebral cortex (CT), cerebellum (CB) and hippocampus (HP) of control rats, and rats killed at the end of the 3 week midazolam (MDZ) treatment. The bars show the mean + S.E.M of four to eight independent experiments for CT and CB, and two experiments for HP (no S.E.M. calculated). Midazolam treatment had no significant effect on the B_{max} or K_d of [^{3}H]zolpidem binding.

(Fig. 5), although the same chronic midazolam treatment resulted in tolerance (Ramsey-Williams et al., 1994).

4. Discussion

The principal findings of the present study were that the B_{max} of [^{3}H]zolpidem binding was decreased in hippocampus and cerebellum after only 1 or 2 week flurazepam treatment, and in these regions as well as in cerebral cortex after 2 week treatment. Acute treatment with desalkyl-flurazepam failed to change [^{3}H]zolpidem binding in any region. Three week midazolam treatment did not change [^{3}H]zolpidem binding. The K_d of [^{3}H]zolpidem binding was not changed except in cerebral cortex after 1 week flurazepam treatment. The decrease in B_{max} of [^{3}H]zolpidem binding in the present study can be contrasted to the results of an earlier study in which 1 and 2 week flurazepam treatments did not change the binding of [^{3}H]-flunitrazepam in cerebral cortical plus hippocampal tissue (Rosenberg and Chiu, 1981a). A more recent study (Wu et al., 1994), in which [^{3}H]zolpidem and [^{3}H]flunitrazepam binding were compared following a 4 week flurazepam treatment, showed that binding of the BZ_1 receptor selective ligand, [^{3}H]zolpidem, was

also decreased in cerebellum, but no change in cerebellar binding of [^{3}H]flunitrazepam was found.

To be sure that changes in [^{3}H]zolpidem binding were a response to chronic benzodiazepine treatment, rats were given a single dose of desalkyl-flurazepam, which is the main active metabolite of flurazepam and several times more potent than flurazepam (Randall and Kappell, 1973; Tyma et al., 1984). It has been shown that a single acute dose of rats with 2.5 mg/kg desalkyl-flurazepam resulted in benzodiazepine activity levels in brain equivalent to that found after 1 week of chronic flurazepam treatment (Xie and Tietz, 1992). In the present work, this treatment failed to alter [^{3}H]zolpidem binding in cerebellum and hippocampus, in which a significant decrease in [^{3}H]zolpidem binding was found after 1 week flurazepam treatment. This result, along with the lack of change in cerebral cortex after only 1 week of flurazepam treatment, showed that down-regulation of the BZ receptor required chronic benzodiazepine exposure. This result also indicated that the decrease in B_{max} of [^{3}H]zolpidem binding after chronic flurazepam treatment did not result from residual drug remaining in the brain tissue after chronic flurazepam treatment, which was also concluded in a previous study (Wu et al., 1994).

Though autoradiographic study (Tietz et al., 1986) showed [^{3}H]flunitrazepam binding was decreased in some discrete regions after a 1 week flurazepam treatment, homogenate binding assays failed to detect changes in [^{3}H]flunitrazepam binding in cerebral cortex (including hippocampus) (Rosenberg and Chiu, 1981a). This discrepancy could be due to the sensitivity of the homogenate binding assay not being sufficient to detect such small, localized changes. However, using the same homogenate binding assay method, decreased [^{3}H]zolpidem binding was detected in all three regions studied after 2 week flurazepam treatment, and even after 1 week flurazepam treatment in cerebellum and hippocampus. These results were in general agreement with a previous study (Wu et al., 1994), in which [^{3}H]zolpidem binding, but not [^{3}H]flunitrazepam binding, was decreased in cerebellum after flurazepam treatment for 4 weeks. Since [^{3}H]zolpidem binding represents the BZ_1 receptor, while [^{3}H]flunitrazepam binding represents both BZ_1 and BZ_2 receptors, a decrease in [^{3}H]zolpidem binding, with no associated change in [^{3}H]flunitrazepam binding, indicated that after 1 week and 2 week flurazepam treatment, the BZ_1 receptor was preferentially decreased. In the case of cerebral cortex and hippocampus, where BZ_1 receptors account for 52% and 34% of the total BZ receptor population (Wu et al., 1994), the changes in BZ_1 binding might not have been detectable with the non-selective ligand, [^{3}H]flunitrazepam, though previous work had found significant down-regulation as small as 12% using this ligand (Rosenberg and Chiu, 1981b). However, in cerebellum, in which BZ_1 receptors account for 91% of the total BZ receptor population (Wu et al., 1994), the down-regulation measured with [^{3}H]zolpidem should have been readily detected with [^{3}H]flunitrazepam. The results in cerebellum supported those of the previous study (Wu et al., 1994), which suggested that BZ receptors that were not labelled by [^{3}H]zolpidem, presumably 'BZ_2 receptors', had been increased so that the [^{3}H]flunitrazepam binding remained unchanged. A similar conclusion was drawn from the results of other work, in which mice were treated chronically with lorazepam. In that study, [^{3}H]flunitrazepam binding in the absence and in the presence of CL 218,872 (to inhibit the BZ_1 binding), showed increased BZ_2 receptor binding, and suggested decreased BZ_1 receptor binding in cortex and hippocampus (Galpern et al., 1990). Thus, the present study, as well as previous results (Galpern et al., 1990; Wu et al., 1994), suggests that BZ receptor subtypes may be differentially regulated, and that those BZ receptors not bound by zolpidem may have been up-regulated, especially in cerebellum. The availability of ligands selective for other subpopulations of BZ receptors might allow a direct evaluation of this possibility.

The decrease in the BZ_1 receptor could be related to those measures of tolerance detected after only a week of flurazepam treatment. These included anticonvulsant tolerance, measured as a decreased ability of benzodiazepines to raise the pentylenetetrazole seizure threshold (Rosenberg et al., 1991), decreased rotational behavior elicited by unilateral microinjection of flurazepam into the substantia nigra pars reticulata (Tietz and Rosenberg, 1988), and reduced benzodiazepine effect to suppress neuronal activity in substantia nigra pars reticulata (Tyma et al., 1988; Rosenberg et al., 1990). Though the loss of [^{3}H]zolpidem binding indicates that changes in the $GABA_A$ receptor have occurred, it can not be used as an explanation for tolerance since diazepam (Wu et al., 1994) and midazolam treatments that cause tolerance are not associated with loss of [^{3}H]zolpidem binding. However, such different effects of chronic treatments on BZ receptor binding may be related to the differing manifestations of tolerance found following chronic treatments with different benzodiazepines (Ramsey-Williams et al., 1994; Rosenberg et al., 1991).

The decrease in BZ_1 receptor number might result from decreased levels of some $GABA_A$ receptor subunit mRNAs which are related to the BZ_1 receptor (e.g. α_1, $\beta_{2,3}$ and γ_2) (Pritchett et al., 1989). A clear relationship between changes in $GABA_A$ receptor subunit mRNA levels during benzodiazepine treatment and receptor regulation has not been well established. In one study, we described the time course and regional localization of a decrease in the level of the γ_2 subunit mRNA in flurazepam-treated rats (Zhao et al.,

1994a). Those results were in agreement with the down-regulation of [^{3}H]flunitrazepam binding previously reported (Rosenberg and Chiu, 1981a,b). Both the γ_2 mRNA level and [^{3}H]flunitrazepam binding were decreased in cerebral cortex and hippocampus, but not in cerebellum, after 4 weeks, but not after 2 weeks of treatment (Rosenberg and Chiu, 1981a,b; Zhao et al., 1994a). Other studies also reported decreases in the levels of various $GABA_A$ receptor subunit mRNAs after chronic benzodiazepine treatment (Heninger et al., 1990; Kang and Miller, 1991; O'Donovan et al., 1992a,b; Primus and Gallager, 1992; Tietz et al., 1994). Since BZ_1 receptor binding sites are thought to be determined by the presence of the α_1 subunit (Pritchett et al., 1989), changes in the mRNA level for this subunit are of particular interest for the present work. In rats treated with diazepam, the α_1 subunit mRNA was reported to be decreased in cerebral cortex, though not in hippocampus or cerebellum (Heninger et al., 1990). In our laboratory, the same diazepam treatment was associated with decreased α_1 mRNA levels in hippocampus only (Wu et al., 1995). However, neither of these reported decreases in α_1 subunit mRNA was associated with any change in binding using various ligands, including the BZ_1 selective agents, DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate) (Heninger and Gallager, 1988) and zolpidem (Wu et al., 1994). In mice treated with lorazepam, the decrease in BZ receptor binding and increase in CL 218,872-resistant (BZ_2) binding was detected on day 7 of chronic treatment (Galpern et al., 1990). Though those results suggested a loss of BZ_1 binding sites, the same lorazepam treatment had no effect on α_1 subunit mRNA in cerebral cortex until at least 14 days of treatment, after which the level was decreased (Kang and Miller, 1991). Thus, the decrease in α_1 subunit mRNA in these studies was not correlated with changes in BZ receptor binding. A similar mismatch exits when considering the decreased [^{3}H]zolpidem binding in the present and previous (Wu et al., 1994) studies. The flurazepam treatment we have used altered the levels of several $GABA_A$ receptor subunit mRNAs, including γ_2 (Zhao et al., 1994a), as well as β_2 and β_3 (Zhao et al., 1994b). However, this flurazepam treatment did not result in measurable changes in α_1 subunit mRNA (Zhao et al., 1994a). One possibility is that Northern analysis lacks sufficient sensitivity. Indeed, an in situ hybridization study using rats 48 h after a week of flurazepam treatment showed localized decreases in α_1 subunit mRNA limited to hippocampal CA1 region and cerebral cortical layers II and IV (Tietz et al., 1994). However, it is not clear that such discretely localized changes could be responsible for the robust decreases in [^{3}H]zolpidem binding in brain homogenates found in the present study. Overall, the studies of $GABA_A$ receptor subunit mRNA levels suggest changes in subunit expression and possibly in receptor turnover, but so far provide little insight into the basis for down-regulation of BZ receptor binding, or the selective loss of BZ_1 and apparent increase in BZ_2 sites.

In contrast to flurazepam treatment, chronic midazolam treatment did not change [^{3}H]zolpidem binding to cerebral cortex, hippocampus or cerebellum, although this treatment did produce tolerance to the anticonvulsant effect of benzodiazepines (Ramsey-Williams et al., 1994). In a previous study, in which the same midazolam treatment was used, no change in [^{3}H]flunitrazepam binding was found in cerebral cortex, hippocampus or cerebellum (Ramsey-Williams et al., 1994). These results suggest that tolerance after chronic midazolam treatment may be associated with changes in $GABA_A$ receptors other than those detected by BZ receptor binding. A similar conclusion was drawn from the results of chronic diazepam treatment (Wu et al., 1994).

In summary, the present study showed the B_{max} of [^{3}H]zolpidem binding to rat brain was decreased after shorter flurazepam treatment, and in more brain regions than could be detected in previous studies with the BZ receptor subtype non-selective ligand, [^{3}H]flunitrazepam. The difference between [^{3}H]-flunitrazepam binding and [^{3}H]zolpidem binding in the previous (Wu et al., 1994) and the present study indicated that BZ receptor subtypes are differentially regulated, and that this regulation may not be detected without the use of appropriately selective ligands.

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