ELSEVIER

European Journal of Pharmacology 295 (1996) 169–179

ejp

An autoradiographic study of [^{3}H]flunitrazepam binding sites in the brain of rat made tolerant to and dependent on pentobarbital

Toshihito Suzuki [a,b], Takehiko Ito [a], Susan E. Wellman [a], Ing Kang Ho [a,*]

[a] *Department of Pharmacology and Toxicology, The University of Mississippi Medical Center, Jackson, MS, USA*
[b] *Department of Psychiatry, Institute of Clinical Medicine, The University of Tsukuba, Tsukuba, Ibaraki, Japan*

Received 27 July 1995; revised 15 September 1995; accepted 19 September 1995

Abstract

The effects of continuous administration of pentobarbital on the benzodiazepine receptor labeled by [^{3}H]flunitrazepam were investigated. Animals were made tolerant to pentobarbital by intracerebroventricular (i.c.v.) infusion with pentobarbital (300 μg/10 μl/h) for 6 days through pre-implanted cannulae connected to osmotic mini-pumps. The dependent rats were assessed 24 h after cessation of pentobarbital infusion. Changes in [^{3}H]flunitrazepam binding were examined in 37 brain regions at a concentration of [^{3}H]flunitrazepam of 1 nM. In subsequent saturation studies, the binding parameters B_{max} and K_D were also investigated in 17 brain regions, most of which showed significant changes in [^{3}H]flunitrazepam binding in experiments using a fixed concentration of radioligand. The pentobarbital-tolerant rats showed a significant increase in B_{max} with an increase in K_D for [^{3}H]flunitrazepam in the ventroposterior nucleus of thalamus. In the dependent rats, a significant increase in B_{max} for [^{3}H]flunitrazepam binding, without a change in K_D, was observed in all layers of the frontal cortex, the caudate-putamen, olfactory tubercle, and some nuclei in thalamus, compared to those in the control. Increased [^{3}H]flunitrazepam binding in the molecular layer of the olfactory bulb, the ventral pallidum, and the cerebellum of the pentobarbital dependent rats at a fixed concentration of [^{3}H]flunitrazepam was also observed. There was no significant change in [^{3}H]flunitrazepam binding in the hippocampus and several nuclei of the brain stem. These findings suggest that benzodiazepine receptors are closely involved in the development of tolerance to and dependence on pentobarbital. Further studies on changes in γ-aminobutyric acid $(GABA)_A$ receptor subunit mRNA or the effects of pentobarbital on $GABA_A$ receptor phosphorylation would be necessary for an explanation of the precise mechanisms underlying the development of tolerance to and dependence on pentobarbital.

Keywords: Pentobarbital; $GABA_A$ receptor; Benzodiazepine binding; Drug tolerance; Substance dependence

1. Introduction

Barbiturates have been widely used as anesthetics, hypnotics, and anticonvulsant drugs. However, prolonged misuse of barbiturates in humans leads to the development of physical dependence, characterized by a severe, life-threatening abstinence syndrome following their abrupt withdrawal (Morgan, 1990). In order to study the pharmacological mechanisms in the brain that underlie these phenomena, investigators have utilized numerous experimental models to induce tolerance to and dependence on barbiturates (Ho and Harris, 1981). The indices used to assess the development of barbiturate tolerance are different among previous studies and depend on the species used. Loss of righting reflex and degree of hypothermia are commonly used for assessing tolerance in many species (Ho and Harris, 1981). Physical dependence, in which barbiturate withdrawal reactions are observed, is characterized by tremors, convulsions, and delirium in severe cases (Ho and Harris, 1981). First twitch and pentylenetetrazol-induced convulsions have been used as indices of dependence on barbiturates (Flint and Ho, 1980; Kimura et al., 1993; Tseng et al., 1993a). To administer drug, we have implanted slow-release pellets (Ito et al., 1989; Saunders et al., 1990; Kimura et al., 1991) or used intracerebroventricular (i.c.v.) infusion (Kimura et al., 1993; Tseng et al., 1993a,b, 1994; Miyaoka et al., 1994) for inducing tolerance to and dependence on pentobarbital. In particular, the model

* Corresponding author. Department of Pharmacology and Toxicology, The University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA. Tel.: (601) 984-1600; fax: (601) 984-1637.

Elsevier Science B.V.
SSDI 0014-2999(95)00595-1

developed using i.c.v. infusion of pentobarbital has the advantage that it avoids induction of drug metabolizing enzymes by barbiturates (Kimura et al., 1993).

GABA and benzodiazepine binding sites are both structurally and functionally coupled. Barbiturates have specific actions on GABA-benzodiazepine receptor complex. In particular, barbiturate tolerance and dependence cause subtle changes in the properties of the GABA-benzodiazepine receptor complexes. The GABAergic neurons were shown to play an important role in the development of barbiturate tolerance and dependence (Saunders and Ho, 1990). In vitro, barbiturates have been demonstrated to enhance benzodiazepine binding in brain homogenates (Leeb-Lundberg et al., 1980; Ticku, 1981; Skolnick et al., 1981; Corda et al., 1988) and in whole brain (Carlson et al., 1992). Changes in benzodiazepine receptors following chronic administration of barbiturates were reported in mice receiving intraperitoneal (i.p.) injections of phenobarbital (Sonawane et al., 1980), or taking foods with phenobarbital added (Liljequist and Tabakoff, 1985), and in rats injected with phenobarbital intraperitoneally (Mohler et al., 1978), with pentobarbital pellet implantation (Saunders et al., 1990), or with intracerebroventricular (i.c.v.) infusion with pentobarbital (Tseng et al., 1993a,b, 1994; Miyaoka et al., 1994). Reported effects of chronic administration of barbiturates on benzodiazepine binding sites (Mohler et al., 1978; Sonawane et al., 1980; Liljequist and Tabakoff, 1985) are inconsistent. The inconsistency may be due to differences in species, assay conditions, and dosing regimens. Regional changes in [^{3}H]flunitrazepam binding have not yet been investigated in detail in animals chronically treated with pentobarbital.

In the present study, we report our investigation of the effects of pentobarbital tolerance and dependence on [^{3}H]flunitrazepam binding sites in various discrete regions of the brain, including those undetectable in our previous studies (Tseng et al., 1993a; Miyaoka et al., 1994), using in vitro receptor autoradiography techniques. Animals were rendered tolerant to and dependent on pentobarbital by the method of i.c.v. infusion of the drug. This experimental procedure has been shown to be an appropriate experimental model for inducing tolerance to and dependence on barbiturates (Kimura et al., 1993; Tseng et al., 1993a).

2. Materials and methods

[^{3}H]Flunitrazepam (85.8 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). All other chemicals used in this study were obtained from Sigma Chemical (St. Louis, MO, USA).

Male Harlan Sprague-Dawley rats (Indianapolis, IN, USA) weighing 225–250 g were used. Animals were maintained on a 12/12-h light-dark cycle at constant temperature, with free access to standard laboratory feed and tap water, for one week before treatment commenced.

The surgery for intracerebroventricular (i.c.v.) infusion of pentobarbital in the rat was performed as described (Kimura et al., 1993; Tseng et al., 1993a,b, 1994; Miyaoka et al., 1994). Briefly, rats were anesthetized with Equithesin (4.25 g chloral hydrate, 2.23 g $MgSO_4 \cdot 7H_2O$, 0.972 g sodium pentobarbital, 44.4 ml propylene glycol, 10 ml 95% ethanol, and distilled water to a final volume of 100 ml), 3 ml/kg i.p. A 21-gauge stainless steel cannula was implanted in the right lateral ventricle (L: 1.3 mm; A-P: − 0.5 mm, and D-P: 4.5 mm) of the rat brain with bregma chosen as the stereotaxic reference point (Paxinos and Watson, 1982). Rats were allowed one week recovery period prior to the pentobarbital treatment. Following the recovery period, animals were infused for 6 days by an osmotic minipump (Alzet 2ML1, Alza, Palo Alto, CA, USA) with pentobarbital (300 μg/10 μl/h) or saline vehicle. The rats made tolerant to pentobarbital were decapitated immediately following the termination of pentobarbital infusion. The rats made dependent on pentobarbital were killed 24 h after the disconnection of osmotic pumps. The control groups with saline infusion were treated in a similar fashion.

Autoradiograms were generated according to the method of Ito et al. (1994). Rats were decapitated and the brains were rapidly removed. After freezing the brain in liquid nitrogen, coronal sections were cut at a thickness of 12 μm on a microtome cryostat and thaw-mounted onto a gelatin-coated slide. Sections were stored at −80°C until the assay.

In preliminary studies for assessing optimal conditions for autoradiography, the characteristics of [^{3}H]flunitrazepam binding sites of the slide-mounted sections were examined in order to obtain the best ratio of total to non-specific binding, yet maintain the highest possible specific binding. The preliminary experiments were performed by the methods of Young and Kuhar (1980) with a minor modification (Miyaoka et al., 1994). The coronal sections at the level of hippocampus, at interaural 6.20 mm, bregma −2.80 mm in a stereotaxic atlas of rat brain (Paxinos and Watson, 1984), containing the parietal cortex, hippocampus, thalamus, and amygdala, were incubated with 1 nM [^{3}H]flunitrazepam in 50 mM Tris-citrate buffer (pH 7.1) with 150 mM NaCl, in the presence or absence of 1 μM flunitrazepam, at room temperature and at 4°C. The slides were then rinsed for various lengths of time in the same buffer, followed by one dipping with distilled water, and rapidly dried. After the wash, the tissues on the slides were quickly wiped off with a Whatman GF/B filter and placed in scintillation vials. The radioactivity was measured by liquid

scintillation counter (Tri-Carb, 2200CA, Packard Instrument Company, IL, USA).

The dissociation of [^{3}H]flunitrazepam from the slide-mounted sections was examined after incubation with 1 nM [^{3}H]flunitrazepam for 60 min at 4°C. The tissue sections were then transferred to the buffer for various lengths of time for up to 60 min. [^{3}H]-Flunitrazepam dissociated from the receptors faster at 4°C than at 22°C. The experiments were repeated 3 times. The best ratio of specific to non-specific binding was at about 10 min. Therefore, we routinely utilized a rinse of 5 min twice for the kinetic and saturation experiments (data not shown). The effect of preincubation on [^{3}H]flunitrazepam binding to tissue sections was studied for up to 30 min in the same buffer at 4°C and 22°C. [^{3}H]Flunitrazepam binding gradually decreased up to 30 min. In addition, we incubated tissue sections of naive rats with those of pentobarbital-treated rats. The results were compared to the data from the tissue sections of naive rats incubated alone, to determine if any residual pentobarbital in the brains of the pentobarbital-treated rats would affect our binding data. We determined that there was no significant difference between the two groups. The percentage of [^{3}H]flunitrazepam binding in tissue sections from naive rats incubated together with tissue sections of pentobarbital-treated rats, relative to that of tissue sections from naive rats incubated alone, was 98% (average from five experiments). The results using tissue sections of pentobarbital-treated rats instead of those from naive rats were similar. Thus, no preincubation was necessary, and preincubation was not used in subsequent experiments. Specific binding of [^{3}H]-flunitrazepam was time-dependent. It reached steady-state after about 90 min of incubation at 4°C in 1 nM [^{3}H]flunitrazepam and after 40 min at 22°C. Although the association rate of [^{3}H]flunitrazepam binding to tissue sections was slower at 4°C than at 22°C, the maximal level of [^{3}H]flunitrazepam binding was higher at 4°C than at 22°C. Our assay conditions were therefore an incubation for 90 min and two rinses for 5 min each, without preincubation.

We analyzed autoradiograms by a densitometer to examine changes in [^{3}H]flunitrazepam binding. Coronal sections were incubated with 1 nM [^{3}H]flunitrazepam for 90 min at 4°C using the assay conditions described above. Non-specific binding observed in the presence of 1 μM flunitrazepam was less than 5% of the corresponding total binding in 1 nM [^{3}H]-flunitrazepam and was negligible for analyzing the autoradiograms. The total binding density in the individual region was considered to be that of specific binding. In the drug titration experiments, the subsequent sections were incubated with concentrations of [^{3}H]flunitrazepam ranging from 0.15 nM to 6.0 nM. Non-specific binding observed in the presence of 1 μM flunitrazepam was approximately 10% of the total binding at a concentration of 6.0 nM [^{3}H]flunitrazepam and specific binding was therefore calculated as the difference between the total binding and the non-specific binding. The tissue sections used for titration studies of [^{3}H]flunitrazepam binding were taken from the frontal level, approximately at interaural 10.00 mm and bregma 1.00 mm, and from the hippocampus level, at interaural 5.40 mm and bregma −3.60 mm in a stereotaxic atlas of rat brain (Paxinos and Watson, 1984). Both sections contained the 17 brain regions studied, e.g. layers I–III, IV, and V–VI of the frontal cortex, the cingulate cortex, anterior part of the caudate-putamen, nucleus accumbens, olfactory tubercle, septum, layers I–III, IV, and V–VI of the parietal cortex, piriform cortex, dentate gyrus and stratum oriens of CA1 field of the hippocampus, globus pallidus, and central medial and ventroposterior nuclei of thalamus.

Dried sections were juxtaposed to tritium-sensitive film (Hyperfilm-^{3}H, Amersham International, Buckinghamshire, UK), together with a commercial tritium standard (ART 123, American Radiolabeled Chemicals, St. Louis, MO, USA), in an exposure holder. The films were exposed at −80°C for 2 weeks. After exposure, the film was developed in Kodak D-19 at a room temperature for 5 min and fixed for 10 min. Autoradiograms were analyzed by a digital scanning densitometer (Molecular Dynamics, Sunnyvale, CA, USA), operating on the image acquisition and analysis program ImageQuant 3.3. (Molecular Dynamics, Sunnyvale, CA, USA). The density in each region was recorded by positioning a circle cursor among 4–18 areas over each region, or by outlining the border of regions on bilateral sides of the brain images, depending on the shape and size of the region examined. Each value was the mean of duplicate determinations. The mean values were determined from 5–8 rats and expressed as the mean ± S.E.M., in fmol/mg wet weight of brain tissue. Anatomical structures of the rat brain were identified from the tissue sections stained with cresyl violet, with reference to the brain atlas defined by Paxinos and Watson (1984).

Scatchard transformation of the data from each region showed a single linear plot. Linear regression analyses were used to obtain values of maximal numbers of binding sites (B_{max}) and dissociation constants (K_D). Analysis of variance (one-way ANOVA) was used to test statistical significance among the four groups. The Newman-Keuls multiple range test was applied for the degree of significance.

3. Results

[^{3}H]Flunitrazepam binding was unevenly distributed throughout the rat brain (Table 1a and 1b). Among 37

Table 1a
The changes in [^{3}H]flunitrazepam binding sites of the discrete brain regions of rats made tolerant to and dependent on pentobarbital

	Control 1	Tolerance	Control 2	Dependence
Olfactory bulb	185.7 ± 8.2	216.6 ± 8.9 [a]	180.7 ± 10.9	223.7 ± 6.0 [b]
Cerebral cortices				
Frontal cortex				
layer I–III	84.6 ± 5.9	95.7 ± 4.7	89.6 ± 3.0	103.9 ± 4.3 [b]
layer IV	96.9 ± 6.5	116.5 ± 4.0 [a]	108.3 ± 3.0	123.2 ± 4.7 [a]
layer V–VI	66.3 ± 5.1	76.4 ± 5.2	70.1 ± 2.7	87.9 ± 3.1 [b]
Cingulate cortex	97.4 ± 6.2	103.8 ± 5.6	102.5 ± 3.6	115.0 ± 5.9
Piriform cortex	77.1 ± 3.7	76.1 ± 2.0	87.3 ± 5.6	86.2 ± 3.9
Parietal cortex				
layer I–III	92.3 ± 5.9	95.6 ± 5.4	107.2 ± 7.1	109.9 ± 5.3
layer IV	109.6 ± 7.7	109.2 ± 6.1	128.2 ± 7.5	128.5 ± 4.8
layer V–VI	79.7 ± 5.9	78.6 ± 3.7	86.3 ± 5.6	92.5 ± 3.5
Entorhinal cortex	76.0 ± 6.6	80.0 ± 4.3	82.0 ± 5.5	90.1 ± 3.2
Retrosplenial	72.5 ± 7.0	78.4 ± 6.2	70.0 ± 3.6	82.0 ± 3.0
Caudate-putamen				
anterior parts	35.6 ± 2.6	42.6 ± 4.1	40.8 ± 3.0	50.9 ± 1.8 [a]
posterior parts	35.7 ± 4.4	36.6 ± 2.3	34.5 ± 1.4	44.4 ± 4.2
Globus pallidus	36.1 ± 2.7	39.8 ± 3.5	39.3 ± 1.3	51.3 ± 4.7 [a]
Ventral pallidum	116.3 ± 4.5	115.6 ± 7.7	113.5 ± 5.0	133.9 ± 4.8 [a]
Accumbens nucleus	43.0 ± 2.9	49.9 ± 4.0	45.7 ± 4.3	53.0 ± 1.0
Olfactory tubercle	67.1 ± 3.3	65.3 ± 3.3	64.8 ± 4.0	76.1 ± 3.3 [a]
Medial septal nucleus	99.4 ± 10.4	107.3 ± 11.0	110.7 ± 8.8	111.3 ± 6.0
Lateral septal nucleus	34.8 ± 6.8	44.8 ± 4.4	42.2 ± 3.0	45.1 ± 3.4
Bed nucleus of stria terminalis	42.1 ± 3.4	39.7 ± 4.2	41.9 ± 2.6	50.4 ± 2.3

Animals were rendered tolerant to and dependent on pentobarbital as described in Materials and methods. The tissue sections were incubated with 1.0 nM [^{3}H]flunitrazepam. Binding capacity is expressed as fmol/mg wet weight of tissue. Each value is the mean of duplicate determinations and represents the mean ± S.E.M. of 5–8 rats. [a] $P < 0.05$ and [b] $P < 0.01$.

Table 1b
The changes in [^{3}H]flunitrazepam binding sites of the discrete brain regions of rats made tolerant to and dependent on pentobarbital

	Control 1	Tolerance	Control 2	Dependence
Hypothalamus				
anterior area	44.5 ± 3.9	50.3 ± 4.8	50.8 ± 7.1	45.5 ± 3.0
ventromedial nucleus	79.4 ± 5.9	78.6 ± 4.2	89.7 ± 4.4	87.1 ± 3.8
Zona incerta	48.7 ± 5.5	52.6 ± 5.0	55.2 ± 1.4	55.5 ± 3.4
Thalamus				
laterodorsal	28.6 ± 4.8	28.4 ± 2.6	33.2 ± 5.1	34.1 ± 5.5
ventroposterior (lateral, medial)	21.1 ± 5.7	25.5 ± 3.5	24.2 ± 4.0	34.4 ± 4.2 [a]
paraventricular	59.5 ± 5.0	65.5 ± 2.4	74.2 ± 4.1	64.1 ± 5.2
Amygdala				
medial basolateral nucleus	74.7 ± 5.9	77.5 ± 3.6	89.5 ± 7.0	82.4 ± 3.9
Hippocampal complex				
stratum oriens of CA1	78.1 ± 3.6	83.9 ± 6.4	90.8 ± 6.2	88.2 ± 5.3
dentate gyrus	91.9 ± 3.1	97.9 ± 6.2	107.3 ± 7.0	107.7 ± 5.7
Superior colliculus				
superficial gray layer	108.3 ± 8.9	116.7 ± 6.4	120.4 ± 13.0	124.1 ± 4.6
Substantia nigra	62.1 ± 10.2	49.4 ± 4.5	48.8 ± 6.9	63.4 ± 6.1
Central gray	67.4 ± 6.6	74.5 ± 4.1	70.4 ± 8.5	77.8 ± 5.8
Sibiculum	64.4 ± 10.0	60.9 ± 3.7	56.9 ± 5.4	66.1 ± 6.2
Presubiculum	85.9 ± 4.5	82.1 ± 4.0	83.5 ± 7.9	90.4 ± 5.2
Inferior colliculus	126.4 ± 6.5	124.7 ± 2.5	116.6 ± 13.4	125.2 ± 7.5
Cerebellum				
molecular layer	54.1 ± 3.2	58.7 ± 3.6	54.6 ± 4.7	65.8 ± 2.6 [a]
granular layer	20.9 ± 6.8	22.7 ± 7.3	28.2 ± 6.8	23.8 ± 5.5

Animals were rendered tolerant to and dependent on pentobarbital as described in Materials and methods. The tissue sections were incubated with 1.0 nM [^{3}H]flunitrazepam. Binding capacity is expressed as fmol/mg wet weight of tissue. Each value is the mean of duplicate determinations and represents the mean ± S.E.M. of 5–8 rats. [a] $P < 0.05$ and [b] $P < 0.01$.

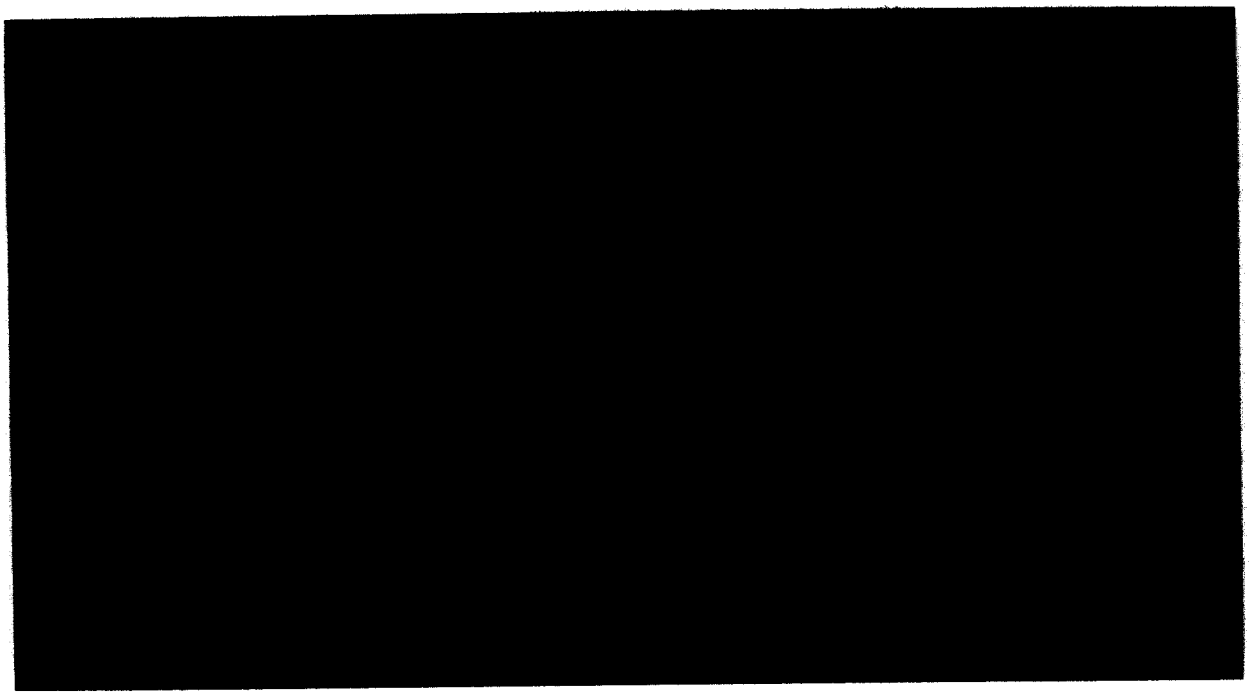


Fig. 1. Autoradiographic images of [^{3}H]flunitrazepam binding sites in rat brains of control (left) and pentobarbital dependence (right).

brain regions studied, the greatest density of specific binding was found in the in the olfactory bulb. Moderately high levels of binding were observed in layer IV of the frontal and parietal cortices, the cingulate cortex, ventral pallidum, medial septum nucleus, dentate gyrus in the hippocampus, and superior and inferior colliculi. The entorhinal cortex and piriform cortex, olfactory tubercle, ventromedial nucleus in the hypothalamus, stratum oriens of CA1 field, and basolateral nucleus of the amygdala displayed moderate levels of [^{3}H]flunitrazepam binding. The caudate-putamen, several nuclei of the thalamus, and the granular layer of the cerebellum showed relatively low densities of [^{3}H]flunitrazepam binding.

Changes in [^{3}H]flunitrazepam binding in the brains of rats made tolerant to and dependent upon pentobarbital were examined in these 37 discrete brain regions. Fig. 1 shows representative autoradiograms of brain sections obtained from the control and dependent rats. As shown in Table 1a and 1b, a significant increase in [^{3}H]flunitrazepam binding was found in layer IV of the frontal cortex (+20%) and in the molecular layer of the olfactory bulb (+17%) in pentobarbital-tolerant rats, as compared with that of the control group. There was, however, no significant change in the other brain regions studied. In the pentobarbital-dependent rats, [^{3}H]flunitrazepam binding was significantly increased in layers I–III (+16%), IV (+14%), and V–VI (+25%) of the frontal cortex, the molecular layer of the olfactory bulb (+24%), and olfactory tubercles (+17%) in the olfactory system, the anterior part of caudate-putamen (+25%), globus pallidus (+31%), ventral pallidum (+18%), the ventroposterior nucleus of the thalamus (41%), and the molecular layer of the cerebellum (21%), as compared with that of the control group. However, no significant changes were found in the brain regions with relatively higher levels of [^{3}H]flunitrazepam binding, e.g., the dentate gyrus and stratum oriens of field CA1 of the hippocampus, cingulate cortex, nucleus accumbens, and superior and inferior colliculi.

In studies of receptor characteristics, the values of the binding parameters B_{max} and K_D in 17 selected discrete brain regions were investigated (Table 2a and 2b). The B_{max} values ranged from 229.8 ± 11.3 fmol/mg tissue weight in the cingulate cortex to 67.0 ± 2.6 fmol/mg tissue weight in the ventroposterior nucleus of the thalamus in the control 1 group. The K_D values ranged from 0.55 ± 0.04 nM in the globus pallidus to 0.92 ± 0.07 nM in the central medial nucleus of the thalamus in the control 1 group. The K_D values in the frontal cortex (layer IV) and hippocampus (CA1) were 0.77 ± 0.05 nM and 0.70 ± 0.07 nM, respectively.

In the pentobarbital-tolerant rats, the B_{max} values of [^{3}H]flunitrazepam binding in ventroposterior nucleus of thalamus were significantly increased (24%), with a significant increase in K_D (49%), as compared with those in the control 1 group. No significant changes in the binding parameters were demonstrated in other brain regions.

The pentobarbital-dependent rats showed a significant increase in B_{max} values with no changes in K_D values in all layers (I–III (14%), IV (14%), V–VI (13%)) of the frontal cortex (Fig. 2a), caudate-putamen

Table 2a
Comparison of the B_{max} values (fmol/mg wet weight of tissue) of [^{3}H]flunitrazepam binding sites in discrete brain regions among tolerant, dependent, and control rats

	Control 1	Tolerant	Control 2	Dependent
Cerebral cortex				
Frontal				
layer I–III	187.8 ± 9.9	197.0 ± 4.8	181.4 ± 8.9	207.3 ± 4.1 [a]
layer IV	224.8 ± 8.8	229.9 ± 6.0	217.2 ± 6.8	248.0 ± 4.0 [a]
layer V–VI	160.4 ± 5.9	169.2 ± 2.9	163.1 ± 9.8	183.9 ± 4.6 [a]
Cingulate	229.8 ± 11.3	236.6 ± 6.9	233.0 ± 12.1	251.9 ± 9.3
Parietal				
layer I–III	185.9 ± 3.6	181.3 ± 6.8	169.0 ± 6.3	178.0 ± 5.9
layer IV	219.4 ± 5.4	218.1 ± 5.5	205.1 ± 4.3	206.5 ± 3.1
layer V–VI	163.2 ± 3.8	164.5 ± 2.1	153.2 ± 4.3	157.6 ± 3.5
Piriform	166.8 ± 6.4	165.4 ± 3.4	152.3 ± 7.9	162.7 ± 8.3
Caudate-putamen	83.4 ± 3.8	81.7 ± 1.4	85.4 ± 6.1	96.8 ± 3.8 [a]
Globus pallidus	116.5 ± 3.5	107.1 ± 5.3	108.5 ± 3.8	123.3 ± 7.4
Nucleus accumbens	121.4 ± 7.2	127.1 ± 4.1	125.2 ± 8.3	130.3 ± 6.7
Septum	134.3 ± 4.5	154.1 ± 11.8	150.9 ± 13.3	143.7 ± 14.8
Olfactory tubercle	126.7 ± 4.4	139.1 ± 3.9	129.1 ± 4.7	152.7 ± 5.7 [a]
Hippocampus				
stratum oriens (CA1)	152.7 ± 8.2	158.3 ± 5.7	145.0 ± 4.6	152.5 ± 5.2
dentate gyrus	185.3 ± 8.7	192.1 ± 5.5	167.4 ± 9.1	185.7 ± 6.8
Thalamus				
ventroposterior	67.0 ± 2.6	83.3 ± 4.2 [a]	57.2 ± 3.2	73.1 ± 3.2 [a]
central medial	136.9 ± 1.9	152.4 ± 7.4	125.3 ± 6.8	142.2 ± 4.0 [a]

Animal were rendered tolerant to and dependent on pentobarbital as described in Materials and methods. Each region of tissue section used in these experiments corresponds to the frontal level approximately at interaural 10.00 mm and bregma 1.00 mm, and to the hippocampal level approximately at interaural 5.40 mm and bregma −3.60 mm from the stereotaxic atlas of Paxinos and Watson. Binding capacity is expressed as fmol/mg wet weight of tissue. Each value is the mean of duplicate determinations and represents the mean ± S.E.M. of 5–8 rats. [a] $P < 0.05$.

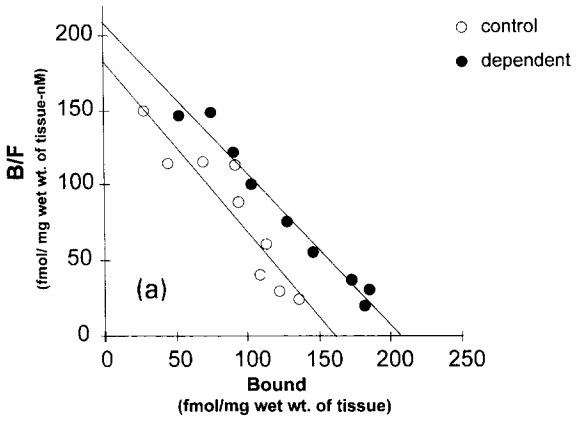
Table 2b
Comparison of the K_D values (nM) of [^{3}H]flunitrazepam binding sites in discrete brain regions among tolerant, dependent, and control rats

	Control 1	Tolerant	Control 2	Dependent
Cerebral cortex				
Frontal				
layer I–III	0.75 ± 0.08	0.80 ± 0.10	0.67 ± 0.05	0.79 ± 0.07
layer IV	0.77 ± 0.05	0.80 ± 0.09	0.71 ± 0.10	0.77 ± 0.04
layer V–VI	0.7 ± 0.08	0.75 ± 0.06	0.68 ± 0.03	0.81 ± 0.06
Cingulate	0.74 ± 0.05	0.83 ± 0.08	0.76 ± 0.05	0.82 ± 0.06
Parietal				
layer I–III	0.79 ± 0.10	0.68 ± 0.05	0.71 ± 0.09	0.63 ± 0.04
layer IV	0.82 ± 0.11	0.76 ± 0.05	0.62 ± 0.06	0.67 ± 0.04
layer V–VI	0.77 ± 0.09	0.75 ± 0.05	0.66 ± 0.07	0.67 ± 0.05
Piriform	0.75 ± 0.12	0.75 ± 0.05	0.64 ± 0.07	0.71 ± 0.06
Caudate-putamen	0.58 ± 0.07	0.84 ± 0.06	0.71 ± 0.08	0.93 ± 0.10
Globus pallidus	0.55 ± 0.04	0.63 ± 0.07	0.56 ± 0.04	0.59 ± 0.03
Nucleus accumbens	0.72 ± 0.07	0.73 ± 0.07	0.76 ± 0.06	0.72 ± 0.06
Septum	0.88 ± 0.08	0.97 ± 0.19	0.89 ± 0.15	0.84 ± 0.07
Olfactory tubercle	0.64 ± 0.05	0.77 ± 0.07	0.64 ± 0.05	0.74 ± 0.07
Hippocampus				
stratum oriens (CA1)	0.70 ± 0.07	0.69 ± 0.04	0.68 ± 0.06	0.65 ± 0.04
dentate gyrus	0.73 ± 0.08	0.76 ± 0.06	0.61 ± 0.06	0.70 ± 0.04
Thalamus				
ventroposterior	0.90 ± 0.18	1.34 ± 0.14 [a]	0.97 ± 0.13	1.09 ± 0.09
central medial	0.92 ± 0.07	0.98 ± 0.06	0.92 ± 0.07	0.90 ± 0.08

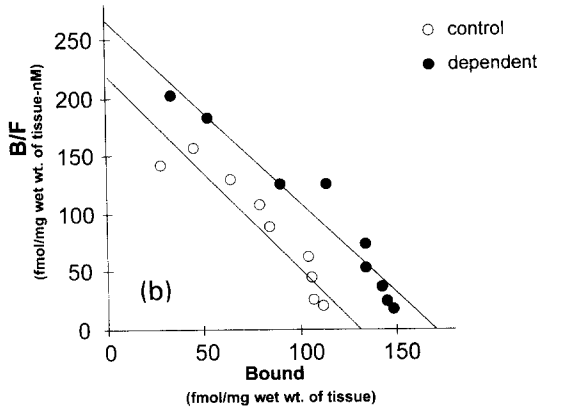
Animals were rendered tolerant to and dependent on pentobarbital as described in Materials and methods. Each region of tissue section used in these experiments corresponds to the frontal level approximately at interaural 10.00 mm and bregma 1.00 mm, and to the hippocampal level approximately at interaural 5.40 mm and bregma −3.60 mm from the stereotaxic atlas of Paxinos and Watson. Binding capacity is expressed as fmol/mg wet weight of tissue. Each value is the mean of duplicate determinations and represents the mean ± S.E.M. of 5–8 rats. [a] $P < 0.05$.

(13%) (Fig. 2b), olfactory tubercle (18%) (Fig. 2c), and ventroposterior nucleus (28%) and central medial nucleus (13%) in thalamus, as compared to those in the control 2 group. No significant changes in B_{max} and K_D values were demonstrated in the other brain regions.

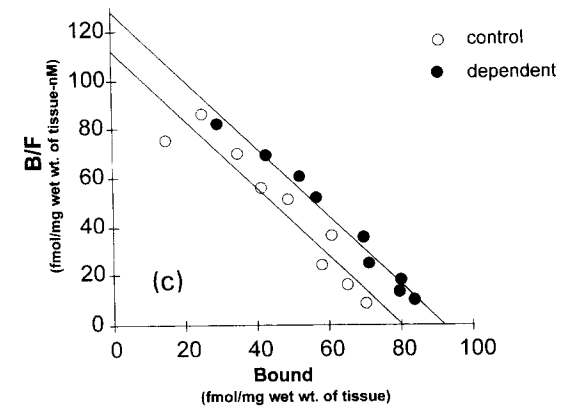
Scatchard plots of [^{3}H]FNZ binding in layer I-III of frontal cortex



Scatchard plots of [^{3}H]FNZ binding in olfactory tubercle



Scatchard plots of [^{3}H]FNZ binding in caudate-putamen



4. Discussion

In order to increase the specific binding of [^{3}H]flunitrazepam, 150 mM NaCl was added to the incubation buffer (Carlson et al., 1992; Miyaoka et al., 1994). Under the assay conditions, binding reached steady-state after 90 min at 4°C; binding capacity appeared to be higher than at 22°C. Preincubation for up to 30 min at 4°C did not significantly increase [^{3}H]flunitrazepam binding. These results were consistent with those reported previously (Young and Kuhar, 1980). The previous kinetic studies of [^{3}H]flunitrazepam binding in rat brains using quantitative autoradiographic techniques (Young and Kuhar, 1980; Carlson et al., 1992) showed approximately 2- or 3-fold higher K_D values than we obtained in our study. The K_D values of [^{3}H]flunitrazepam binding in rat brain homogenates (Tseng et al., 1993a; Miyaoka et al., 1994) are also greater than those we reported. Cuatrecasas and Hollenberg (1976) pointed out that apparent K_D values are dependent on the total receptor concentration used in the binding study. Apparent K_D values for [^{3}H]quinuclidinyl benzilate were found to be increased as the thickness of the tissue section was increased (Nonaka and Moroji, 1984). Furthermore, K_D values depended on the duration of incubation time in autoradiographic studies and were decreased as incubation time was increased (Nonaka and Moroji, 1985). In a kinetic study of [^{3}H]cholecystokinin (CCK)-8 binding sites, K_D values obtained in brain homogenates were also greater than those in brain tissue sections (Sekiguchi and Moroji, 1986). We used thinner sections, 12 μm, and our studies employed longer incubation times, in comparison to conditions used in previous experiments (Young and Kuhar, 1980; Carlson et al., 1992). The differences in apparent K_D values may be due to these differences in assay conditions. The pattern of [^{3}H]flunitrazepam distribution in the brain that we observed was consistent with those of

Fig. 2. Representative Scatchard plots of [^{3}H]flunitrazepam ([^{3}H]FNZ) binding to the frontal cortex (Fig. 2a), caudate-putamen (Fig. 2b), olfactory tubercle (Fig. 2c). The titration experiments were carried out with [^{3}H]flunitrazepam concentrations ranging from 0.15 nM to 6.0 nM. Open and solid circles indicate [^{3}H]flunitrazepam binding in control and pentobarbital-dependent rats, respectively. Barbiturate tolerance and dependence cause subtle changes in the properties of the GABA-benzodiazepine receptor complexes. The GABAergic neurons play an important role in the mechanisms involved in barbiturate tolerance and dependence (Saunders and Ho, 1990). These brain regions have mainly functional roles for regulating behavior and emotional state rather than physiological functions (Koob, 1992). The region-specific changes in our study are of interest since the regional distributions are different from that with great changes in [^{3}H]flunitrazepam binding in the presence of pentobarbital in vitro (Carlson et al., 1992).

previous reports (Young and Kuhar, 1980; Daval et al., 1991; Carlson et al., 1992; Sola et al., 1993).

In pentobarbital-tolerant rats, no significant differences in the densities of [^{3}H]flunitrazepam binding sites incubated at a fixed concentration (1 nM) of [^{3}H]flunitrazepam were observed in most regions, except for layer IV of the frontal cortex and the molecular layer of the olfactory bulb. Scatchard analysis of the data revealed no changes in B_{max} and K_D values in any regions, even in layer IV of the frontal cortex. Thus changes in binding characteristics in the present study were not necessarily reflected in experiments done using a fixed concentration of radioligand. In addition, the B_{max} value for [^{3}H]flunitrazepam was significantly increased in the ventroposterior nucleus of the thalamus, while there was no change in the density of [^{3}H]flunitrazepam binding in a single point measurement (1nM [^{3}H]flunitrazepam) in this area. This apparent discrepancy seems, at least in part, to be due to an increased K_D value (i.e. lower affinity) for [^{3}H]flunitrazepam binding. Other experiments, for example kinetic studies, might be more useful in assessing changes in binding sites. Contradictory results regarding the effects of chronic administration of barbiturates on benzodiazepine receptors have been reported (Mohler et al., 1978; Sonawane et al., 1980; Liljequist and Tabakoff, 1985; Saunders et al., 1990; Tseng et al., 1993a; Miyaoka et al., 1994). No changes in [^{3}H]diazepam binding were reported after chronic phenobarbital treatment in SPF rats (Mohler et al., 1978). Our recent studies in brain homogenates also revealed no significant changes in either B_{max} or K_D values of [^{3}H]flunitrazepam binding sites in SD rats (Tseng et al., 1993a; Miyaoka et al., 1994). Liljequist and Tabakoff (1985) reported, however, that chronic phenobarbital treatment decreased B_{max} values with no change in K_D value for [^{3}H]flunitrazepam in the cortex and cerebellum of C57B1 mice in brain homogenates. Sonawane et al. (1980) demonstrated a decrease in B_{max} and K_D values for [^{3}H]diazepam in whole brains of CD-1 mice treated with phenobarbital. The discrepancies among these studies may be attributed to many factors, including varied barbiturate concentrations in brain tissues, the experimental procedures for making animals tolerant to barbiturates, the species used, and assay conditions.

In the pentobarbital-dependent rats, the binding sites labeled by [^{3}H]flunitrazepam were increased in a relatively large number of brain regions, e.g. in layers I–III, IV, and V–VI of the frontal cortex, the olfactory systems (olfactory bulb, olfactory tubercles), the caudate-putamen, the ventral pallidum, the globus pallidus, the ventroposterior nucleus in the thalamus, and the molecular layer of the cerebellum, in the tissue sections incubated with 1 nM [^{3}H]flunitrazepam. No significant changes were noted in the other brain regions that are rich in [^{3}H]flunitrazepam binding sites, e.g. the stratum oriens of CA1 field and the dentate gyrus in the hippocampus, the cingulate cortex, and the superior and inferior colliculi. Scatchard analysis of the drug titration data also revealed an increase in [^{3}H]flunitrazepam binding in all these brain regions except in the globus pallidus. This suggests that an increase in [^{3}H]flunitrazepam binding resulted from an increase in B_{max} values with no change in K_D. The discrepancy in the results for globus pallidus may be due to a difference in the level of coronal brain sections, which were used at an anterior level (interaural 8.60 mm, bregma −0.40 mm) for analysis at a fixed concentration, and at a posterior level (interaural 6.70 mm, bregma −2.30 mm) for titration with drug and Scatchard analysis, respectively. Increased binding capacity of [^{3}H]flunitrazepam with no change in affinity in membrane preparations from frontal cortex, striatum, and cerebellum in pentobarbital-dependent rats has been reported (Tseng et al., 1993a; Miyaoka et al., 1994). Our results suggest that the changes in [^{3}H]flunitrazepam binding occur in more brain regions than previously reported in pentobarbital-dependent rats. Miyaoka et al. (1994) demonstrated that the B_{max} values for binding of [^{3}H]Ro15-1788 (flumazenil), an antagonist at benzodiazepine receptors, was unchanged 24 h after pentobarbital withdrawal, and that 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), which affects the lipid constituents of biological membranes, caused a decrease in B_{max} and K_D values in membranes isolated after withdrawal, indicating that pentobarbital withdrawal induces some conformational changes in benzodiazepine receptors, resulting in an increase in B_{max} for [^{3}H]flunitrazepam binding. For a high affinity [^{3}H]muscimol site, Tseng et al. (1993a) showed an increase in the maximum binding capacity (B_{max}) in the frontal cortex and an increased K_D value in the cerebellum in pentobarbital-dependent rats. $GABA_B$ binding sites labeled by [^{3}H]baclofen also increased, with an elevation of K_D, in frontal cortex, while no significant change was observed in the cerebellum (Kimura et al., 1991). Considering the inhibitory effect of $GABA_B$ receptors at presynaptic neurons on GABA release, it appears that changes in the $GABA_B$ receptor may in part be related to up-regulation of presynaptic $GABA_A$ receptors. It was proposed that the ability of GTP binding proteins to interact with $GABA_B$ receptors was altered by pentobarbital treatment, causing changes in the $GABA_B$ receptor (Kimura et al., 1991). Chronic pentobarbital treatment also caused an increase in *t*-[^{35}S]butylbicyclophosphorothionate (TBPS) binding, which occurs at a site on the chloride channel of $GABA_A$-benzodiazepine receptor complex, in the frontal cortex, the substantia nigra, and the cerebellum of pentobarbital-dependent rats (Ito et al., 1989; Tseng et al., 1993a).

The changes in [^{35}S]TBPS binding in the substantia nigra are probably related to the increased susceptibility to seizures induced by pentylenetetrazol. In the present study, an increase of [^{3}H]flunitrazepam binding was seen in the centromedial nucleus in the thalamus in pentobarbital-dependent rats. The central medial nucleus in thalamus has been reported to play an important role in regulating seizures induced by pentylenetetrazol (Miller et al., 1990; Miller and Ferrendelli, 1990). Taken together with the evidence of inhibition of GABAergic afferent in the thalamus from the substantia nigra (Gale, 1985), these data may explain in part the mechanisms through which increased sensitivity to pentylenetetrazol-induced seizure develops in pentobarbital-dependent rats. Other work indicates that some differences in region-specific changes among $GABA_A$, $GABA_B$, and benzodiazepine receptors are observed in pentobarbital-dependent rats (Ito et al., 1989; Saunders et al., 1990; Tseng et al., 1993a; Miyaoka et al., 1994). This suggests that each of the binding sites on the $GABA_A$-benzodiazepine receptor complex changes in a different manner in both tolerance and dependence.

Our data support the idea that the changes in benzodiazepine receptor are associated with the pathophysiology of pentobarbital dependence in various brain regions, e.g., the frontal cortex, limbic system, striatum, and cerebellum, mediated directly and/or indirectly by GABAergic neural transmission. Previous work from our laboratory has shown that there are changes in [^{3}H]flunitrazepam binding sites in the frontal cortex, cerebellum, and striatum in pentobarbital dependence. These data show that changes in B_{max} occur, particularly in all layers of the frontal cortex, the molecular layer of cerebellum, and the anterior part of the striatum. Also, the cerebral cortices, other than frontal cortex, showed no significant changes in binding parameters in pentobarbital dependence in our study. This raises the possibility that the involvement of $GABA_A$-benzodiazepine receptors is different in the development of pentobarbital dependence. Based on the affinity for different benzodiazepines, two types of benzodiazepine receptors have been identified in the brain, type I, with a high affinity for triazolopyridazine or β-carboline-3-carboxylate, and type II, with a low affinity for these compounds (Klepner et al., 1979; Squires et al., 1979). Most brain regions contain both type I and type II sites to varying degrees. Preferential enrichment in type I sites has been demonstrated in the cerebral cortex, globus pallidus, thalamus, substantia nigra, and cerebellum. Type II sites are predominantly in the cortex, hippocampus (particularly the dentate gyrus), caudate-putamen, nucleus accumbens, and amygdala (Niddam et al., 1987; Langer et al., 1990; Doble and Martin, 1992). However, it is still difficult to correlate benzodiazepine subtype selectivity with a dissociated behavioral profile (Doble and Martin, 1992). Considering the characteristic distributions of benzodiazepine type I and II sites, significant changes in [^{3}H]flunitrazepam binding observed in the present study occur in both benzodiazepine type I and II sites. Niehoff et al. (1983) showed that pentobarbital preferentially stimulated benzodiazepine type I receptors in both cerebral cortex and cerebellum. It was subsequently demonstrated that chronic phenobarbital administration caused a decrease of benzodiazepine type I in cerebellum and of benzodiazepine type II in cerebral cortex in mice (Liljequist and Tabakoff, 1985). Although our results showed a change in [^{3}H]-flunitrazepam binding in only the pentobarbital-dependent state, both benzodiazepine type I and II sites are involved in the pharmacological changes induced by chronic treatment with barbiturates. These brain regions are primarily involved in regulating behavior and emotional state rather than physiological functions (Koob, 1992). The region-specific changes that we observed here are of interest, since the regions that show changes differ from those that showed substantial changes in [^{3}H]flunitrazepam binding in the presence of in vitro pentobarbital (Carlson et al., 1992).

In view of the characteristic functions and regional distribution of $GABA_A$ receptor subunit mRNA, benzodiazepine type II sites probably represent a heterogeneous population of sites containing α_2, α_3, and α_5 subunits of $GABA_A$ receptors, while type I sites closely correspond to receptors containing the α_1 subunit (Pritchett et al., 1989b; Pritchett and Seeburg, 1990; Luddens and Wisden, 1991; Doble and Martin, 1992). The regional distribution of γ_2 subunit mRNA is reported to correspond to that obtained from autroradiographic studies for benzodiazepine receptors (Olsen et al., 1990). In particular, γ_2 subunit mRNA is closely involved in the pharmacological functions of benzodiazepine (Pritchett et al., 1989a). Pentobarbital can fully activate a $GABA_A$ receptor channel of the subunit combination $\alpha_1\beta_1\gamma_2$ (Malherbe et al., 1990). Supporting evidence is an increase in α_1- and γ_2-subunit mRNA of $GABA_A$ receptor in all layers of neocortex and piriform cortices, and in granule and Purkinje cell layers of the cerebellum in pentobarbital-dependent rats (Tseng et al., 1993b, 1994). $GABA_A$ receptor β_3-subunit mRNA levels also are altered in neocortex (Tseng et al., 1994). These findings suggest the changes in [^{3}H]flunitrazepam binding in rats made dependent upon pentobarbital may be responsible for certain physiological dysfunctions of dependence, and may result from alterations in the $GABA_A$ molecular subunits rather than subtype selectivity of benzodiazepine. There are, however, some discrepancies between regional alterations in the receptor binding and changes in subunit mRNA levels in pentobarbital dependence. Recently, Sanna et al. (1995) reported that direct ac-

tion of pentobarbital is observed with homomeric β_1 $GABA_A$ receptor subunit. It suggests a specific subunit of $GABA_A$ receptor contains sites for the direct activating effect of pentobarbital. Thus, additional studies of changes in other subunit mRNAs of $GABA_A$ receptors are necessary to elucidate the mechanisms underlying the development of pentobarbital tolerance and dependence.

Evidence that $GABA_A$ receptors are phosphorylated by several protein kinases has accumulated (Browning et al., 1990; Leidenheimer et al., 1991, 1993). Second messenger system modulators also regulate the functions of $GABA_A$ receptors ([^{3}H]SR 95531) and a site on chloride channel ([^{35}S]TBPS) in a phosphorylation-dependent manner (Leidenheimer et al., 1990; Lanius et al., 1993). Although the exact mechanism underlying the effect of pentobarbital remains unknown, an inhibitory effect of barbiturates on modulators of second messenger systems, e.g., protein kinase C and forskolin-stimulated adenylate cyclase activity, has been reported (Mikawa et al., 1990; Deshmukh et al., 1989, 1991). Moreover, recent studies suggested that chronic pentobarbital administration produced a decrease in the maximal responses in Cl^- uptake by pentobarbital (Morrow et al., 1990; Allan et al., 1992). Thus, an alteration in second messenger systems following continuous administration of pentobarbital may be involved in the changes in $GABA_A$-benzodiazepine receptor complexes and in Cl^- flux in pentobarbital tolerance and dependence. Further studies of phosphorylation of $GABA_A$ receptors are needed before a definitive statement can be made concerning its role in pentobarbital dependence and tolerance.

In conclusion, we examined the regional changes in benzodiazepine receptors labeled by [^{3}H]flunitrazepam throughout the brains of rats rendered tolerant to and dependent on pentobarbital. Our results suggested that an increase in [^{3}H]flunitrazepam binding with no alteration in the binding affinity occurs in various brain regions and is involved in the development of pentobarbital dependence.

Acknowledgements

The authors thank Mr. J.G. Bennett for his technical assisstance. This work was supported by NIDA Grant No. 04480.

References

Allan, A.M., X. Zhang and L.D. Baier, 1992, Barbiturate tolerance: effects on GABA-operated chloride channel function, Brain Res. 588, 255.

Browning, M.D., M. Bureau, E.M. Dudek and R.W. Olsen, 1990, Protein kinase C and cAMP-dependent protein kinase phosphorylate the β subunit of the purified γ-aminobutyric $acid_A$ receptor, Proc. Natl. Acad. Sci. USA 87, 1315.

Carlson, B.X., A.M. Mans, R.A. Hawkins and H.A. Baghdoyan, 1992, Pentobarbital-enhanced [^{3}H]flunitrazepam binding throughout the rat brain: an autoradiographic study, J. Pharmacol. Exp. Ther. 263, 1401.

Corda, M.G., O. Giorgi, B. Longoni, E. Ongini, A. Barnett, S. Montaldo and G. Biggio, 1988, γ-Aminobutyric acid and pentobarbital enhance 2-[^{3}H]oxoquazepam binding to type I benzodiazepine recognition sites in rat and human brain, J. Neurochem. 50, 681.

Cuatrecasas, P. and M.D. Hollenberg, 1976, Membrane receptors and hormone action, in: Advances in Protein Chemistry, Vol. 30, eds. C.B. Anfinsen, J.T. Edsall and F.M. Richards (Academic Press, New York) p. 251.

Daval, J.L., M.C. Werck, A. Nehlig and A.P. De Vasconcelos, 1991, Quantitative autoradiographic study of the postnatal development of benzodiazepine binding sites and their coupling to GABA receptors in the rat brain, Int. J. Dev. Neurosci. 9, 307.

Deshmukh, D.S., S. Kuizon, V.P.S. Chauhan and H. Brockerhoff, 1989, Effect of barbiturates on polyphosphoinositide biosynthesis and protein kinase C activity in synaptosomes, Neuropharmacology 28, 1317.

Deshmukh, D.S., S. Kuizon, V.P.S. Chauhan and H. Brockerhoff, 1991, Interaction of anesthetic barbiturates with the phosphoinositide-dependent pathway of signal transduction, Ann. NY Acad. Sci. 625, 427.

Doble, A. and I.L. Martin, 1992, Multiple benzodiazepine receptors: no reason for anxiety, Trends Pharmacol. Sci. 13, 76.

Flint, B.A. and I.K. Ho, 1980, Assessment of tolerance to and physical dependence on pentobarbital, induced by multiple pellet implantation, Eur. J. Pharmacol. 65, 355.

Gale, K., 1985, Mechanisms of seizure control mediated by γ-aminobutyric acid: role of the substantia nigra, Fed. Proc. 44, 2414.

Ho, I.K. and R.A. Harris, 1981, Mechanism of action of barbiturates, Annu. Rev. Pharmacol. Toxicol. 21, 83.

Ito, Y., P.A. Saunders, D.K. Lim and I.K. Ho, 1989, Binding characteristics of t-[^{35}S]butylbicyclophosphorothionate in discrete brain regions of rats made tolerant to and dependent on pentobarbital, J. Neurochem. 52, 1093.

Ito, T., T. Suzuki, S.E. Wellman and I.K. Ho, 1995, A novel quantitative receptor autoradiography and in situ hybridization histochemistry technique using storage phosphor screen imaging, J. Neurosci. Methods 59, 265.

Kimura, T., P.A. Saunders, I. Yamamoto and I.K. Ho, 1991, Effects of pentobarbital tolerance to and dependence on $GABA_B$ receptor binding, Neurochem. Res. 16, 1133.

Kimura, T., T. Miyaoka, P.A. Saunders, M.L. Baker, A.S. Hume, I. Yamamoto and I.K. Ho, 1993, Induction of tolerance to and physical dependence on pentobarbital continuous intracerebroventricular administration, J. Pharmacol. Exp. Ther. 266, 1300.

Klepner, C.A., A.S. Lippa, D.I. Benson, M.C. Sano and B. Beer, 1979, Resolution of two biochemically and pharmacologically distinct benzodiazepine receptors, Pharmacol. Biochem. Behav. 11, 457.

Koob, G.F., 1992, Drugs of abuse: anatomy, pharmacology and function of reward pathways, Trends Pharmacol. Sci. 13, 177.

Langer, S.Z., S. Arbilla, J. Benavides and B. Scatton, 1990, Zolpidem and alpidem: two imidazopyridines with selectivity for ω_1- and ω_3-receptor subtypes, in: GABA and Benzodiazepine Receptor Subtypes, eds. G. Biggio and E. Costa (Raven Press, New York) p. 61.

Lanius, R.A., B.A. Pasqualotto and C.A. Shaw, 1993, γ-Aminobu-

tyric acid$_A$ receptor regulation by a chloride-dependent kinase and a sodium-dependent phosphatase, Mol. Brain Res. 20, 192.

Leeb-Lundberg, F., A. Snowman and R.W. Olsen, 1980, Barbiturate receptor sites are coupled to benzodiazepine receptors, Proc. Natl. Acad. Sci. USA 77, 7468.

Leidenheimer, N.J., M.D. Browning, T.V. Dunwiddie, L.D. Hahner and R.A. Harris, 1990, Phosphorylation-independent effects of second messenger system modulators on γ-aminobutyric acid$_A$ receptor complex function, Mol. Pharmacol. 38, 823.

Leidenheimer, N.J., M.D. Browning and R.A. Harris, 1991, GABA$_A$ receptor phosphorylation: multiple sites, actions and artifacts, Trends Pharmacol. Sci. 12, 84.

Leidenheimer, N.J., P.J. Whiting and R.A. Harris, 1993, Activation of calcium-phospholipid-dependent protein kinase enhances benzodiazepine and barbiturate potentiation of the GABA$_A$ receptor, J. Neurochem. 60, 1972.

Liljequist, S. and B. Tabakoff, 1985, Binding characteristics of ^{3}H-flunitrazepam and CL-218,872 in cerebellum and cortex of C57Bl mice made tolerant to and dependent on phenobarbital or ethanol, Alcohol 2, 215.

Luddens, H. and W. Wisden, 1991, Function and pharmacology of multiple GABA$_A$ receptor subunits, Trends Pharmacol. Sci. 12, 49.

Malherbe, P., E. Sigel, R. Baur, E. Persohn, J.G. Richards and H. Mohler, 1990, Functional characteristics and sites of gene expression of the $\alpha_1\beta_1\gamma_2$-isoform of the rat GABA$_A$-receptor, J. Neurosci. 10, 2230.

Mikawa, K., N. Maekawa, H. Hoshina, O. Tanaka, J. Shirakawa, R. Goto, H. Obara and M. Kusunoki, 1990, Inhibitory effect of barbiturates and local anaesthetics on protein kinase C activation, J. Int. Med. Res. 18, 153.

Miller, J.W. and J.A. Ferrendelli, 1990, Characterization of GABAergic seizure regulation in the midline thalamus, Neuropharmacology 29, 649.

Miller, J.W., C.M. Hall, K.D. Holland and J.A. Ferrendeli, 1990, Identification of a median thalamic system regulating seizures and arousal, Epilepsia 30, 493.

Miyaoka, T., T. Kimura, P.A. Saunders, Y.T. Tseng and I.K. Ho, 1994, Binding characteristics of [^{3}H]flunitrazepam in pentobarbital-withdrawal rats, Neurochem. Res. 19, 37.

Mohler, H., T. Okada and S.J. Enna, 1978, Benzodiazepine and neurotransmitter receptor binding in rat brain after chronic administration of diazepam or phenobarbital, Brain Res. 156, 391.

Morgan, W.W., 1990, Abuse liability of barbiturates and other sedative-hypnotics, Adv. Alcohol Subst. Abuse 9, 67.

Morrow, A.L., P. Montpied, A. Lingford-Hughes and S.M. Paul, 1990, Chronic ethanol and pentobarbital administration in the rat: effects on GABA$_A$ receptor function and expression in brain, Alcohol 7, 237.

Niddam, R., A. Dubois, B. Scatton, S. Arbilla and S.Z. Langer, 1987, Autoradiographic localization of [^{3}H]zolpidem binding sites in the rat CNS: comparison with the distribution of [^{3}H]flunitrazepam binding sites, J. Neurochem. 49, 890.

Niehoff, D.L., R.D. Mashal and M.J. Kuhar, 1983, Benzodiazepine receptors: preferential stimulation of type 1 receptors by pentobarbital, Eur. J. Pharmacol. 92, 131.

Nonaka, R. and T. Moroji, 1984, Quantitative autoradiography of muscarinic cholinergic receptors in the rat brain, Brain Res. 296, 295.

Nonaka, R. and T. Moroji, 1985, Time-dependent changes in the binding parameters of muscarinic cholinergic receptors in the rat brain, Brain Res. 326, 379.

Olsen, R.W., R.T. McCabe and J.K. Wamsley, 1990, GABA$_A$ receptor subtypes: autoradiographic comparison of GABA, benzodiazepine, and convulsant binding sites in the rat central nervous system, J. Chem. Neuroanat. 3, 59.

Paxinos, G. and C. Watson, 1982, The Rat Brain in Stereotaxic Coordinates (Academic Press, New York).

Pritchett, D.B. and P.H. Seeburg, 1990, γ-Aminobutyric acid$_A$ receptor α_5-subunit creates novel type II benzodiazepine receptor pharmacology, J. Neurochem. 54, 1802.

Pritchett, D.B., H. Sontheimer, B.D. Shivers, S. Ymer, H. Kettenmann, P.R. Schofield and P.H. Seeburg, 1989a, Importance of a novel GABA$_A$ receptor subunit for benzodiazepine pharmacology, Nature 338, 582.

Pritchett, D.B., H. Luddens and P.H. Seeburg, 1989b, Type I and type II GABA$_A$-benzodiazepine receptors produced in transfected cells, Science 245, 1389.

Sanna, E., F. Garau and R.A. Harris, 1995, Novel properties of homomeric β_1 γ-aminobutyric acid type A receptors: actions of the anesthetics propofol and pentobarbital, Mol. Pharmacol. 47, 213.

Saunders, P.A. and I.K. Ho, 1990, Barbiturates and the GABA$_A$ receptor complex, Prog. Drug Res. 34, 261.

Saunders, P.A., Y. Ito, M.L. Baker, A.S. Hume and I.K. Ho, 1990, Pentobarbital tolerance and withdrawal: correlation with effects on the GABA$_A$ receptor, Pharmacol. Biochem. Behav. 37, 343.

Sekiguchi, R. and T. Moroji, 1986, A comparative study on characterization and distribution of cholecystokinin binding sites among the rat, mouse and guinea pig brain, Brain Res. 399, 271.

Skolnick, P., V. Moncada, J.L. Barker and S.M. Paul, 1981, Pentobarbital: dual actions to increase brain benzodiazepine receptor affinity, Science 211, 1448.

Sola, C., E. Martinez, L. Camon, A. Pazos and E. Rodriguez-Farre, 1993, Lindane administration to the rat induces modifications in the regional cerebral binding of [^{3}H]muscimol, [^{3}H]flunitrazepam, and *t*-[^{35}S]butylbicyclophosphorothionate: an autoradiographic study, J. Neurochem. 60, 1821.

Sonawane, B.R., S.J. Yaffe and B.H. Shapiro, 1980, Changes in mouse brain diazepam receptor binding after phenobarbital administration, Life Sci. 27, 1335.

Squires, R.F., D.I. Benson, C. Baestrup, J. Coupet, C.A. Klepner, V. Myers and B. Beer, 1979, Some properties of brain specific benzodiazepine receptors: new evidence for multiple receptors, Pharmacol. Biochem. Behav. 10, 825.

Ticku, M.K., 1981, Interaction of depressant, convulsant, and anticonvulsant barbiturates with the [^{3}H]diazepam binding site of the benzodiazepine-GABA-receptor-ionophore complex, Biochem. Pharmacol. 30, 1573.

Tseng, Y.T., T. Miyaoka and I.K. Ho, 1993a, Regional-specific changes of GABA$_A$ receptors by tolerance to and dependence upon pentobarbital, Eur. J. Pharmacol. 236, 23.

Tseng, Y.T., S.E. Wellman and I.K. Ho, 1993b, Differential effects on GABA$_A$ receptor γ_2-subunit messenger RNA by tolerance to and withdrawal from pentobarbital – an in situ hybridization study, Life Sci. 53, 321.

Tseng, Y.T., S.E. Wellman and I.K. Ho, 1994, In situ hybridization evidence of differential modulation by pentobarbital of GABA$_A$ receptor α_1- and β_3-subunit mRNAs, J. Neurochem. 63, 301.

Young III, W.S. and M.J. Kuhar, 1980, Radiohistochemical localization of benzodiazepine receptors in rat brain, J. Pharmacol. Exp. Ther. 212, 337.