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Autoradiographic study of [^{3}H]flunitrazepam binding sites in the subnuclei of the thalamus of rats rendered tolerant to and dependent on pentobarbital

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Abstract

We examined changes in benzodiazepine binding sites labeled by [^{3}H]flunitrazepam in five nuclei of the thalamus, the central medial, central lateral, intermediodorsal, ventroposterior, and laterodorsal nuclei, in rats made tolerant to and dependent on pentobarbital. Animals were made tolerant by intracerebroventricular infusion with pentobarbital (300 μg (10 μl)$^{-1}$ h^{-1} for six days) through pre-implanted cannulae. Pentobarbital dependence was assessed 24 h after abrupt withdrawal from pentobarbital. Pentobarbital-tolerant rats showed no significant change in [^{3}H]flunitrazepam binding sites (B_{max} and K_d) in any nucleus examined in the thalamus. In the rats made dependent on pentobarbital, significant increases in the B_{max} of [^{3}H]flunitrazepam binding without changes in K_d were noted in central medial and central lateral nuclei. GABAergic (γ-aminobutyric acid) neurons in the ventrobasal nucleus and in nuclei in the midline group are important in seizure regulation and arousal. These findings suggest that alterations of benzodiazepine receptors in certain nuclei of thalami are involved in the physiological changes induced by pentobarbital dependence. There were no changes in the binding parameters for [^{3}H]flunitrazepam in pentobarbital-tolerant rats.

Keywords: Pentobarbital; Tolerance; Dependence; Benzodiazepine; Thalamus

1. Introduction

The effects of chronic administration of pentobarbital on $GABA_A$ (γ-aminobutyric $acid_A$)–benzodiazepine receptor complexes have been examined using continuous intracerebroventricular (i.c.v.) infusion (Kimura et al., 1993; Tseng et al., 1993a,b, 1994; Miyaoka et al., 1994; Suzuki et al., 1996). Development of tolerance to and dependence on pentobarbital was measured at the end of infusion, whereas physical dependence on pentobarbital was assessed 24 h after discontinuation of the infusion. This procedure induces tolerance to and dependence upon pentobarbital with less chance of hepatic enzyme induction, because peripheral administration of pentobarbital may increase the rate of metabolism in the liver, resulting in a decrease in the half-life of pentobarbital (Kimura et al., 1993). In animals rendered tolerant to pentobarbital, a decrease in duration of the loss of righting reflex (the sleeping time, defined as the time between the animal's loss of righting reflex and the time the animal rights itself spontaneously) and in the degree of hypothermia was observed (Flint and Ho, 1980; Ito et al., 1989; Kimura et al., 1993). These behavioral results indicate a decrease in the potency of pentobarbital due to the development of tolerance to the drug. In pentobarbital-dependent rats, an increased susceptibility to seizures induced by systemically administered pentylenetetrazol and an increase in rectal temperature were evident (Flint and Ho, 1980; Ito et al., 1989; Kimura et al., 1993). These findings suggest that dependence on pentobarbital developed in these animals. Barbiturate tolerance and dependence cause subtle changes in the properties of the $GABA_A$–benzodiazepine receptor complexes. This animal model has been used in biochemical studies on different $GABA_A$ receptor ligands, [^{3}H]muscimol, [^{3}H]flunitrazepam and *t*-[^{35}S]butylbicy-

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clophosphorothionate ([^{35}S]TBPS). Especially, saturation analysis of benzodiazepine sites revealed an increase of binding sites labeled by [^{3}H]flunitrazepam in the frontal cortex, striatum and cerebellum of dependent rats, whereas no change was observed in tolerant rats (for review, see Ito et al., 1996). Tolerance to pentobarbital induces substantial alterations in $\alpha 1$ subunit mRNA in the hippocampus and inferior colliculus. In the dependent rats, there are increases in $\alpha 1$ and $\gamma 2$ subunit mRNAs in the cerebral cortex and cerebellum. These findings indicate that changes in the GABAergic neural transmission occur mainly in the intrinsic neurons throughout the brain, especially in the cerebral cortices, striatum, hippocampal complexes, and cerebellum. The GABAergic neurons were shown to play an important role in the development of barbiturate tolerance and dependence (Ho and Harris, 1981; Saunders and Ho, 1990).

There is increasing evidence to suggest that GABAergic innervation and $GABA_A$–benzodiazepine receptors are in the thalamus (Young and Kuhar, 1980; Bentivoglio et al., 1993). $GABA_A$–benzodiazepine receptors are predominantly distributed in the limbic thalamus, primarily the nuclei of midline group and some adjacent intralaminar nuclei. Although a high concentration of $GABA_A$–benzodiazepine receptor complexes, as for example as in the limbic thalamus, has been shown to be associated with the regulation of anxiety (Reiman et al., 1986), recent studies have suggested that one role of the nuclei of the midline group is to regulate seizure propagation (Miller et al., 1989; Miller and Ferrendelli, 1990a,b). In addition, inhibition of the afferent GABAergic pathway in the thalamus from the substantia nigra was suggested to modulate the propagation of seizure discharges (Gale, 1985). Ito et al. (1989) have previously reported an increase in [^{35}S]TBPS binding in substantia nigra of pentobarbital-dependent rats, which may be involved in the development of increased susceptibility to seizures. In addition, there is supporting evidence for significant changes in the maximal binding labeled by [^{3}H]flunitrazepam in the certain nuclei of the thalamus (Suzuki et al., 1996). In order to further elucidate the biochemical changes in the thalamus, in the study presented here, we examined the changes in benzodiazepine receptors labeled by [^{3}H]flunitrazepam in specific nuclei of the thalamus in rats made tolerant to and dependent on pentobarbital.

2. Materials and methods

2.1. Chemicals

[^{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 (St. Louis, MO, USA).

2.2. Animals

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 225–250 g were used. Animals were maintained on a 12-h light/12-h dark cycle and at constant temperature with free access to standard laboratory feed and tap water for 1 week before treatment commenced.

2.3. Surgical operations

The surgery for i.c.v. infusion of pentobarbital in the rat was performed as previously described (Kimura et al., 1993; Tseng et al., 1993a,b, 1994; Miyaoka et al., 1994; Suzuki et al., 1996). Briefly, rats were anesthetized with Equithesin (4.25 g chloral hydrate, 2.33 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 mg/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 the bregma as the stereotaxic reference point (Paxinos and Watson, 1982). The rats were allowed 1-week recovery prior to the pentobarbital treatment. Following the recovery period, the animals were infused with pentobarbital (300 μg (10 μl)$^{-1}$ h^{-1}) or saline vehicle with an osmotic minipump (Alzet 2ML1, Alza, Palo Alto, CA, USA) for 6 days. The rats made tolerant to pentobarbital were decapitated immediately following the termination of pentobarbital infusion. The rats made dependent upon pentobarbital were killed 24 h after disconnection of the osmotic pumps. The control groups with saline infusion were treated similarly. Control 1 group was the control for the tolerant rats and control 2 group was that for the dependent rats. No significant weight loss in the pentobarbital-treated rats was observed compared to those in the control group.

2.4. Receptor autoradiography

Autoradiography of [^{3}H]flunitrazepam binding sites was carried out according to our methods described earlier (Ito et al., 1995; Suzuki et al., 1996). The rats were decapitated and the brains were rapidly removed. After freezing of the brain at −80°C 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. The assay conditions were those of Young and Kuhar (1980) with minor modifications (Miyaoka et al., 1994). In the saturation experiments, the coronal sections were incubated with concentrations of [^{3}H]flunitrazepam ranging from 0.15 nM to 10.0 nM, for 90 min at 4°C in 50 mM Tris citrate buffer (pH 7.1) with 150 mM NaCl. Adjacent sections were incubated in the presence of 1 μM flunitrazepam to assess nonspecific binding. The slides were then washed twice, five min each, at 4°C in the same buffer, followed by one dip into

distilled water, and rapidly dried. We chose the washing method of Young and Kuhar (1980) in these experiments, although this type of washing will decrease the specific [^{3}H]flunitrazepam binding by a large percentage because of the fast dissociation under these conditions. Dried sections were juxtaposed on tritium-sensitive film (Hyperfilm-^{3}H, Amersham International, Amersham, UK), together with a commercial tritium standard (ART 123, American Radiolabeled Chemicals, St. Louis, MO), 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 room temperature for 5 min and fixed for 10 min.

2.5. Imaging

Autoradiograms were analyzed by means of digital scanning densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA, USA) operating on an image

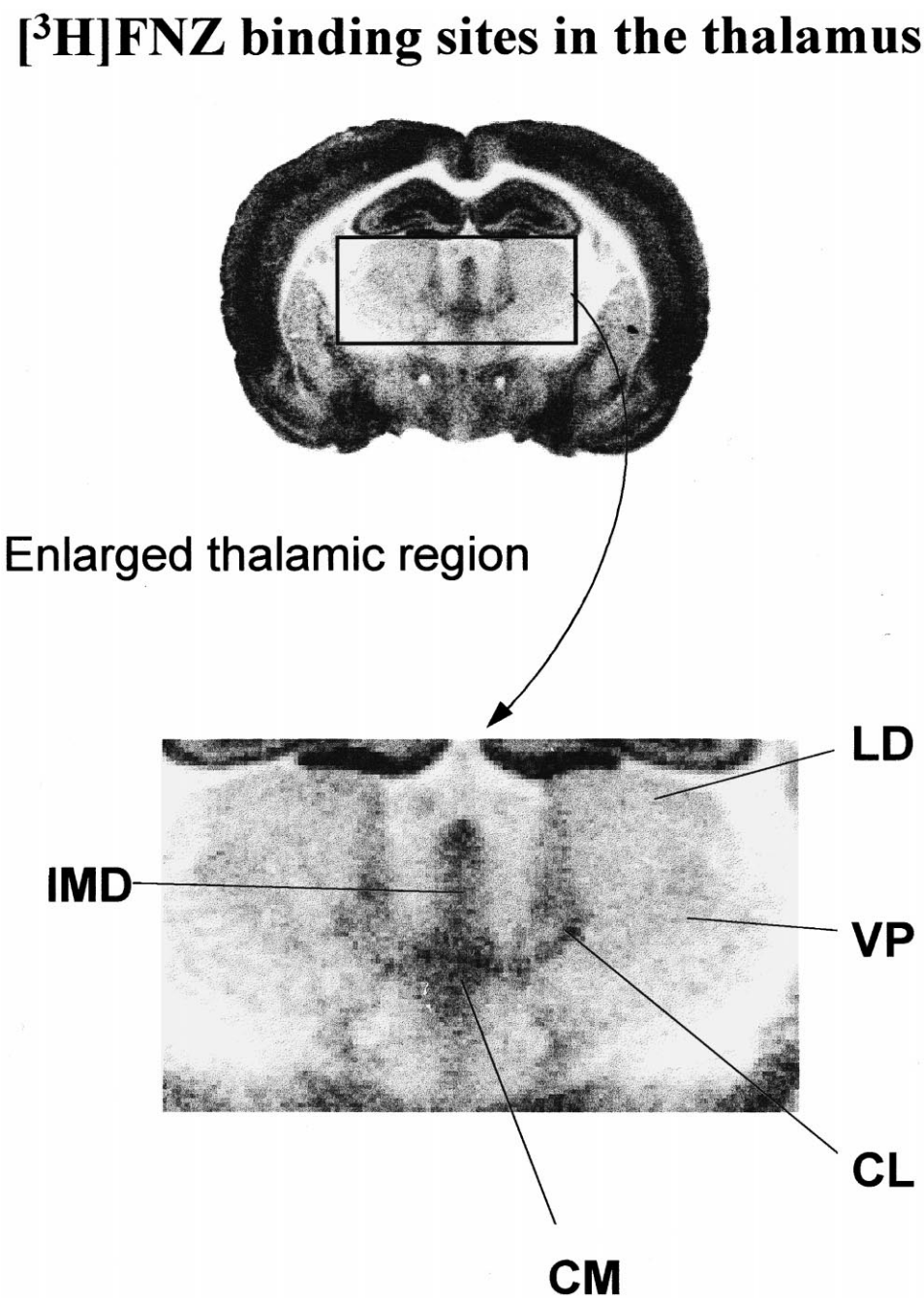

Fig. 1. [^{3}H]flunitrazepam binding sites in the thalamus. Distribution of [^{3}H]flunitrazepam binding sites in a coronal tissue section at the level of the thalamus of rat brain. The autoradiogram corresponded approximately to a level of the Bregma, −3.14 mm and Interaural 5.86 mm. CM, central medial; CL, central lateral; IMD, intermediodorsal; LD, laterodorsal; VP, ventroposterior nuclei.

Table 1
Changes in the B_{max} values of [^{3}H]flunitrazepam binding in the subnuclei of the thalamus

	Control 1	Tolerance	Control 2	Dependence
CM	137.3 (133.4–141.4)	147.9 (141.8–154.2)	125.2 (121.3–129.2)	141.6[a] (138.7–144.5)
CL	107.7 (103.4–112.2)	121.2 (116.7–125.9)	92.6 (84.9–100.8)	112.2[a] (108.8–115.7)
IMD	121.8 (116.1–127.6)	139.6 (133.7–145.9)	118.0 (113.4–122.9)	128.7 (123.0–134.7)
LD	76.7 (73.4–80.2)	88.2 (82.6–94.1)	67.5 (61.9–73.7)	78.9 (76.6–81.2)
VP	67.4 (64.0–70.9)	67.6 (64.4–70.9)	57.9 (54.2–61.8)	70.5 (68.2–72.8)

Animals were made tolerant to and dependent on pentobarbital as described in the text. Units of B_{max} are femtomole per milligram wet weight of tissue. Each value is the mean of five to eight experiments. The data shown in parentheses are 68% confidence interval of the mean of five to eight experiments. Control 1 and control 2 refer to the control groups for the tolerant rats and for the dependent rats, respectively.
[a] $P < 0.05$, differs from each control group.
See Fig. 1 for abbreviations.

acquisition and analysis program (ImageQuant version 3.3, Molecular Dynamics). Nonspecific binding observed in the presence of 1 μM flunitrazepam was less than 10% of the corresponding total binding and was subtracted from its corresponding total image. Specific binding was taken as the difference between total binding and nonspecific binding. Five nuclei of the thalamus, e.g., the central medial, central lateral, intermediodorsal, laterodorsal, and ventroposterior (lateral and medial) nuclei, were examined in the coronal tissue sections, corresponding approximately to a bregma level −3.14 mm and interaural 5.86 mm in the stereotaxic atlas of Paxinos and Watson (1982). The density in each region was recorded by outlining the border of images that corresponded to the desired region on bilateral sides of the brain. Anatomical structures of the rat brain were identified in tissue sections stained with Cresyl violet, with reference to the brain atlas of Paxinos and Watson (1982). Each value was the average of duplicate determinations from a single experiment.

2.6. Data analysis and statistics

Scatchard transformation of the binding data from each region showed a single binding site plot. Nonlinear regression analysis was used to obtain all values of the dissociation constant (K_d) and maximal number of binding sites (B_{max}). Each value was determined from five to eight rats and represents the geometric mean and approximately 68% confidence interval of the mean, in femtomole per milligram wet weight of brain. Statistical analysis was carried out using a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test after log conversion of the raw data.

3. Results

3.1. Distribution and binding characteristics of [^{3}H] flunitrazepam binding in the thalamus

The levels of [^{3}H]flunitrazepam binding were relatively low throughout the thalamus (Fig. 1). Highest concentrations were found in the midline nuclei, e.g., the central medial, central lateral, and intermediodorsal nuclei. In contrast, the laterodorsal and ventroposterior nuclei displayed moderately low concentrations of [^{3}H]flunitrazepam binding. The mean B_{max} values ranged from 137.3 fmol/mg tissue weight in the central medial nucleus to 67.4 fmol/mg tissue weight in the ventroposterior nucleus, among the five nuclei of the thalamus in the control 1 group (Table 1). Relative densities (percentage of binding of each region versus the binding in the central medial nucleus, which had the highest value among the regions examined) in the control 1 group were 78.4% in the central lateral nucleus, 88.7% in the intermediodorsal nucleus, 55.9% in the laterodorsal nucleus, and 49.1% in the ven-

Table 2
Changes in the K_d values of [^{3}H]flunitrazepam binding in the subnuclei of the thalamus

	Control 1	Tolerance	Control 2	Dependence
CM	0.82 (0.80–0.85)	0.92 (0.86–0.99)	0.92 (0.85–0.98)	0.84 (0.76–0.92)
CL	0.89 (0.78–1.00)	0.97 (0.88–1.07)	0.91 (0.81–1.01)	0.85 (0.79–0.93)
IMD	0.79 (0.78–0.80)	1.05 (0.97–1.13)	1.06 (0.95–1.17)	1.00 (0.91–1.09)
LD	0.96 (0.83–1.11)	1.20 (1.11–1.31)	1.05 (0.93–0.19)	1.01 (0.94–1.10)
VP	0.92 (0.75–1.12)	0.92 (0.81–1.05)	0.83 (0.71–0.98)	0.96 (0.91–1.02)

Animals were made tolerant to and dependent upon pentobarbital as described in the text. Units of K_d are nM. Each value is the mean of five to eight experiments. The data shown in parentheses are 68% confidence interval of the mean of five to eight experiments. Control 1 and control 2 refer to the control groups for the tolerant rats and for the dependent rats, respectively. See Fig. 1 for abbreviations.

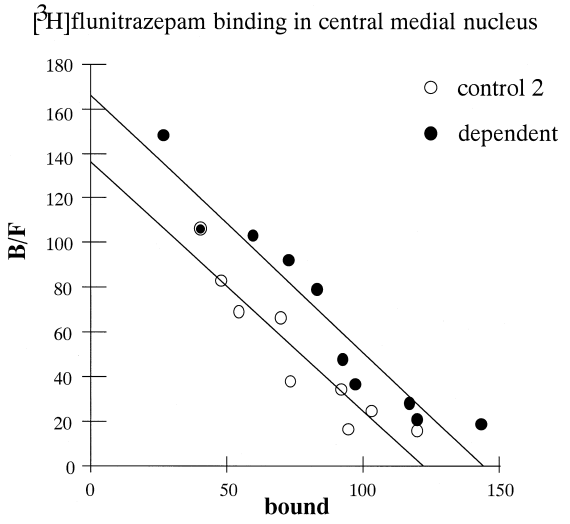

Fig. 2. [^{3}H]flunitrazepam binding in central medial nucleus. Representative Scatchard plots of [^{3}H]flunitrazepam binding to the central medial nucleus in thalamus. The titration experiments were carried out with [^{3}H]flunitrazepam binding concentrations ranging from 0.15 nM to 6.0 nM. Open and solid circles indicate [^{3}H]flunitrazepam binding in control and pentobarbital-dependent rats, respectively.

troposterior nucleus, respectively. The mean K_d values ranged from 0.96 nM in the laterodorsal nucleus to 0.79 nM in the intermediodorsal nucleus in the control 1 group (Table 2). No significant difference in the K_d values was demonstrated among the nuclei assayed.

3.2. B_{max} and K_d values in the pentobarbital-tolerant and pentobarbital-dependent rats

In the pentobarbital-tolerant rats, no significant changes in either B_{max} or K_d values were observed in any nuclei in the thalamus, as compared to those of the corresponding controls (Tables 1 and 2).

Pentobarbital-dependent rats displayed a significant increase in the B_{max} values in the central medial ($+13\%$, $P = 0.016$), central lateral ($+21\%$, $P = 0.012$) thalamic nuclei in comparison with those in the corresponding control group, while the other nuclei examined showed no significant differences in the B_{max} values (Table 1, Fig. 2). There were no significant changes in K_d values in any nuclei examined from pentobarbital-dependent rats (Table 2).

4. Discussion

The observed pattern of distribution of [^{3}H]flunitrazepam binding sites within the thalamus was consistent with that reported by others (Young and Kuhar, 1980; Carlson et al., 1992; Solá et al., 1993). Intrinsic GABAergic cells are innervated in the dorsal thalamus and the reticular nucleus in the thalamus (Bentivoglio et al., 1993). GABAergic innervation is also derived from extrinsic nuclei. For example, ventromedial and mediodorsal regions receive GABAergic afferents from the pars reticularis of the substantia nigra (nigrothalamic afferents) and globus pallidus (pallido-thalamic afferents) (Bentivoglio et al., 1993). High-affinity GABA receptors predominate in the anterior and mediodorsal nuclei. High levels of benzodiazepine receptors, which are allosterically coupled to the GABA receptor, occur in the nuclei of the midline group (Carlson et al., 1992). Although the $GABA_A$–benzodiazepine receptor complex, as mentioned above, is speculated to have functional significance in this area, available experimental evidence is insufficient to delineate its roles. Recent reports suggested that GABA-mediated neural transmission in restricted central medial nuclei was involved in the regulation of seizures (Miller et al., 1989; Miller and Ferrendelli, 1990a,b). Direct and indirect $GABA_A$ receptor agonists, piperidine-4-sulfonic acid and pentobarbital, respectively, infused in the midline thalamus, suppressed tonic seizures and arousal, and yet facilitated myoclonic and clonic seizures induced by pentylenetetrazol (Miller and Ferrendelli, 1990a). These findings suggest that the nuclei of the midline group in the thalamus are some of the important areas responsible for the pentylenetetrazol-induced seizures. In this study, we demonstrated an increase of [^{3}H]flunitrazepam binding sites in the midline nuclei in the thalamus, e.g., central medial and central lateral nuclei, in pentobarbital-dependent rats. Taken together, these results support the notion that upregulation of benzodiazepine receptors in midline nuclei following chronic exposure to pentobarbital resulted in the abnormal sensitivity to pentylenetetrazol-induced seizures. Our earlier report indicated that an increase in *t*-[^{35}S]TBPS sites in the substantia nigra and frontal cortex was correlated with the hypersensitivity to pentylenetetrazol-induced convulsions that occurred in rats dependent on pentobarbital (Ito et al., 1989). Available evidence indicates the presence of GABAergic nigrothalamic afferents in the thalamus (Gale, 1985). It is proposed that the thalamic neurons are part of the seizure circuit that can be regulated by nigra projection (Gale, 1985). Thus, functional changes in the $GABA_A$–benzodiazepine complexes in the thalamus may be involved in the mechanisms of pentylenetetrazol-induced seizures in pentobarbital dependent rats.

In the present study, the ventroposterior nucleus exhibited no significant change in B_{max} in the rats made dependent on pentobarbital, although we had previously detected a change in the binding parameters in the ventroposterior region, using the same ligands as now studied (Suzuki et al., 1996). This discrepancy might be responsible for the difference in brain concentrations of pentobarbital in the rats in the two experiments. Recent evidence suggests that the $GABA_A$–benzodiazepine receptor complex in the ventrobasal nucleus is associated with sensory systems, e.g., the pain system (Roberts et al., 1992; Vogt et al., 1993).

This complex is also the principal relay nuclei for nociceptor-derived information from the spinal cord through a spinothalamic tract (Vogt et al., 1993). In animal experiments, intrathecal injection of pentobarbital produces antinociceptive effects (Ding et al., 1990). Thus, continuous i.c.v. infusion of pentobarbital may affect the binding characteristics of benzodiazepine receptors in the ventroposterior nucleus. Further studies may be needed to elucidate the change in benzodiazepine receptor binding in the ventroposterior nucleus.

Results of our previous study suggested that the changes in benzodiazepine receptors are associated with the pathophysiology of pentobarbital dependence in various brain regions, e.g., the frontal cortex, limbic system, striatum and cerebellum (Suzuki et al., 1996). Moreover, there is supporting evidence that an increase in α_1-, β_3- and γ_2-subunit mRNA of the $GABA_A$ receptor in cerebral cortex and cerebellum in pentobarbital-dependent rats (Tseng et al., 1993b, 1994). In the thalamus, no significant alteration of these mRNA subunits was observed in the laterodorsal and lateral posterior nuclei in pentobarbital-dependent rats (Tseng et al., 1993b). No change in mRNA subunits in the midline nuclei of the thalamus was detected, whereas these regions showed an increase in B_{max} of [^{3}H]flunitrazepam binding sites in pentobarbital-dependent rats in the present study. Nevertheless, it appears that the alteration of benzodiazepine binding in the thalamus as well as in the other brain regions may be mediated directly and/or indirectly by GABAergic neural transmission.

The GABA system has been implicated in a variety of pathological conditions, including seizure control, pain, movement disorders and drug dependence (Ticku, 1983; Ito et al., 1995). The GABAergic neurons are dense in some nuclei of the thalamus. These nuclei play an important role in the regulation of seizures and polymodal sensory systems. However, the precise functions of the $GABA_A$–benzodiazepine receptor complex relating to the physiological roles of the thalamus remain unclear. Moderately high levels of the binding of [^{35}S]TBPS and [^{3}H]zolpidem, a novel imidazopyridine having a preferential affinity for the benzodiazepine1 receptor, are observed in central medial and central lateral nuclei in the thalamus, while most regions of the thalamus contain low densities labeled by the above two radioligands (Niddam et al., 1987; Edgar and Schwartz, 1990; Duncan et al., 1995). The alteration in [^{3}H]flunitrazepam binding in the present study may occur in benzodiazepine-1 receptors rather than in brain areas enriched in benzodiazepine-2 receptors. Further studies are needed to investigate the changes in $GABA_A$ receptor subtypes and subunit mRNAs in the thalamus after chronic exposure to pentobarbital. Electrophysiological studies on the GABAergic neurons in the thalamus would also help to investigate the effect of pentobarbital on $GABA_A$–benzodiazepine receptor complexes in the thalamus.

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