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Benzodiazepine Receptors Increase in Post-Mortem Brain of Chronic Schizophrenics

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Summary. [^{3}H]-Flunitrazepam (FNT) binding was measured in the post-mortem brains of 13 chronic schizophrenics and 10 controls whose mean ages and death-to-freezing intervals were the same in each group. The specific binding of [^{3}H]-FNT to the medial frontal cortex, orbitofrontal cortex, orbital cortex, medial and inferior temporal gyri, superior temporal gyrus, cornu Ammonis 1-3 and putamen was significantly higher in schizophrenics than in controls. Specific binding to the eye movement area (frontal eye field), motor cortex, lateral occipitotemporal gyrus, dentate gyrus of the hippocampus and secondary and tertiary visual cortex did not differ in the two groups. Type 1 benzodiazepine (BZ) binding sites in the superior temporal gyrus of schizophrenics, determined from the displacement of [^{3}H]-FNT binding using a triazolopyridazine, CL 218,872 (200 n*M*), were significantly higher than in the control group. The increase in type 2 BZ binding sites was not significant. Antipsychotic or benzodiazepine medication did not appear to affect the results. There were significant correlations between specific [^{3}H]-FNT binding and concentration of GABA (positive) and of glutamic acid (negative), specific [^{3}H]-kainic acid binding (positive), activity of tyrosine hydroxylase (positive), and substance P-like immunoreactivity (positive) in many areas of the brain. The B_{max} of [^{3}H]-spiperone binding in the putamen was also correlated positively with specific [^{3}H]-FNT binding. These data suggest that dysfunction of BZ receptors may be involved in the pathogenesis and some symptoms of chronic schizophrenia.

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Key words: Chronic schizophrenia – Post-mortem brain – Specific [^{3}H]-flunitrazepam binding – Benzodiazepine receptors – Multiple receptors – CL 218,872 – Neurotransmitters

Introduction

Since the identification of benzodiazepine (BZ) receptors in the rat brain by Squires and Braestrup (1977), investigations have established the pharmacological characteristics of BZ receptors in the mammalian brain (Möhler and Okada 1977; Mackerer et al. 1978). It has been confirmed that most BZ receptors are coupled with GABA receptors and chloride channels (Braestrup and Nielsen 1982). It has been proposed that two subtypes of BZ receptors, type 1 and type 2, are present in the central nervous system (Klepner et al. 1979; Squires et al. 1979; Niehoff and Whitehouse 1983). On the other hand, several candidates for endogenous ligands for BZ receptors, such as β-carbolines and some peptides, including diazepam binding inhibitor (DBI), have been isolated from several mammalian species, including man, and reported to be generally anxiogenic and proconvulsant (Guidotti et al. 1983; Ferrero et al. 1986; Martin 1987; Saano 1987). There have been some reports of a low density of BZ receptors in "fearful" or "emotional" rats and mice (Robertson 1979). Thus, it may be possible to explain the biological bases of pathological anxiety in terms of BZ receptors and their endogenous ligands.

Acute symptoms of schizophrenia can be regarded as hyperdopaminergic states because of the antidopaminergic properties of antipsychotic drugs

(Snyder 1982). Chronic states of the disease, however, must involve many other factors that have never been examined. Therefore, we have measured neurotransmitters, receptors and neuropeptides in the post-mortem brains of chronic schizophrenics (Toru et al. 1982, 1988; Nishikawa et al. 1983).

Anxiety is a symptom most frequently observed in schizophrenia. Therefore, we measured specific [^{3}H]-flunitrazepam (FNT) binding in 12 areas of the post-mortem brain of chronic schizophrenics in the present study. Specific [^{3}H]-FNT binding to type 1 and type 2 BZ binding sites in the superior temporal gyrus was also measured using CL 218,872, a triazolopyridazine that has a preferential affinity for type 1 binding sites (Klepner et al. 1979; Squires et al. 1979; Niehoff and Whitehouse 1983).

Subjects and Methods

Subjects. The post-mortem brains of 10 controls (7 men and 3 women, mean age 66.7 ± 2.7, range 52–74 years) with no history of neuropsychiatric disease and 13 chronic schizophrenic patients (8 men and 5 women, mean age 60.1 ± 3.0, range 41–75 years) were used in this study (Table 1). The ages and

Table 1. Clinical features of schizophrenic patients (S) and controls (C)

	Age	Sex	Type (DSM-III)	Cause of death	Psychotropic medication
A. Schizophrenics					
S1	50	M	Disorganized	Myocardial infarction	Chlordiazepoxide, 10 mg for 7.5 months
S2	62	F	Undifferentiated	Myocardial infarction	Chlordiazepoxide, 10 mg; phenobarbital, 60 mg for 5 days before death
S3	41	M	Catatonic	Pneumonia	Nitrazepam, 10 mg; glutethimide, 500 mg; amitriptyline, 10 mg, for 3 months before death
S5	46	F	Paranoid	Sudden death	Spiperone, 6 mg; pipamperone, 200 mg; thioproperazine, 30 mg; trihexyphenidyl, 8 mg; hydroxyzine, 200 mg, until death
S6	54	M	Undifferentiated	Panperitonitis	Chlorpromazine, 50 mg, until 5 days before death
S7	64	F	Disorganized	Cardiac thrombosis	Haloperidol, 4.5 mg; amitriptyline, 30 mg; promethazine, 75 mg, until 2 days before death
S11	70	M	Disorganized	Myocardial infarction	Drug-free for 7 years
S12	69	M	Disorganized	Cardiac failure	Haloperidol, 3 mg; trihexyphenidyl, 3 mg, until just death
S13	73	M	Undifferentiated	Stomach cancer	Haloperidol, 3 mg; trihexyphenidyl, 3 mg, until 5.5 months; diazepam, 5 mg, until 1.5 months before death
S14	53	F	Disorganized	Ovarian cancer	Oxypertine, 90 mg; clocapramine, 200 mg; amitriptyline, 50 mg, until 15 days before death
S15	68	M	Undifferentiated	Suffocation	Haloperidol, 3 mg; levomepromazine, 25 mg; promethazine, 25 mg; nitrazepam, 10 mg, until just before death
S16	75	F	Disorganized	Pancreatic cancer	Haloperidol, 1.5 mg, until 40 days; clotiazepam, 20 mg, until 33 days before death
S17	56	M	Disorganized	Pneumonia	Chlorpromazine, 300 mg; promethazine, 50 mg, until 35 days before death
B. Controls					
C1	53	M		Sudden death	
C3	67	M		Ileus	
C4	73	M		Pancreatic cancer; D.I. tract bleeding	
C5	52	F		Breast cancer	
C7	71	F		Renal and heart failure	
C11	73	M		Colon cancer	
C12	61	M		Lung cancer, renal failure	
C14	74	F		Pancreatic cancer	
C15	69	M		Common bile duct cancer	
C16	74	M		Disseminated intravascular coagulation	

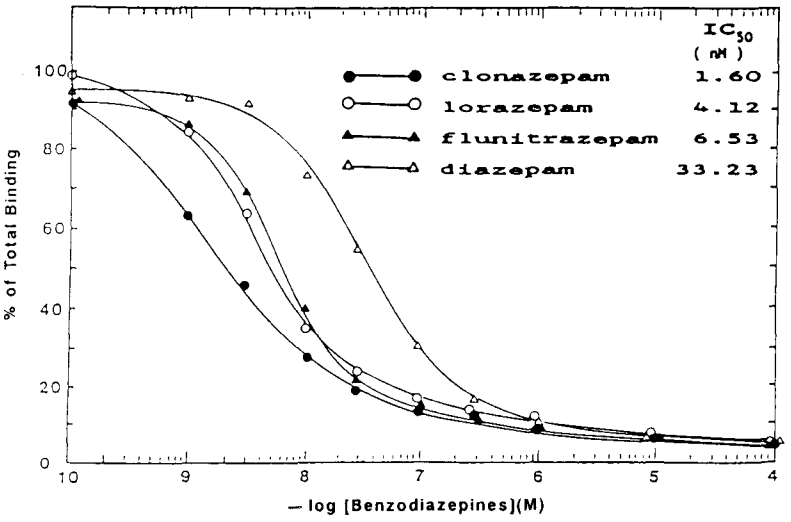

Fig. 1. Inhibition of [^{3}H]-FNT binding to the medial and inferior temporal gyri from a control patient by benzodiazepines. [^{3}]-FNT (85 Ci/mmol) was used in this study (1 n*M*). Drugs were diluted with the buffer from 3×10^{-3} *M* alcohol solution

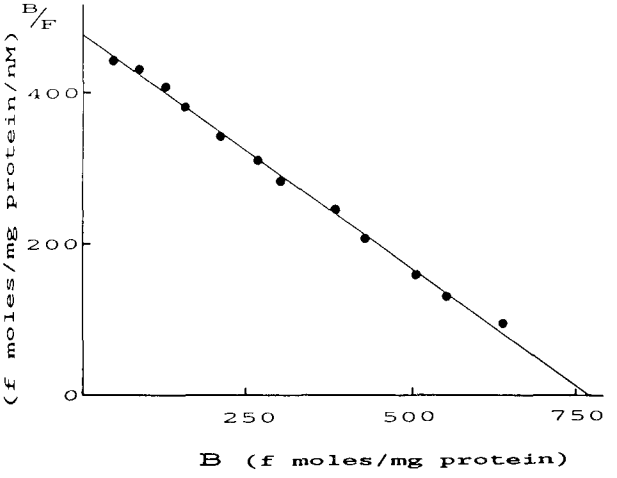

Fig. 2. Scatchard analysis of [^{3}H]-FNT binding to the medial and inferior temporal gyri from a control patient. [^{3}H]-FNT (85 Ci/mmol) ranged from 0.1 to 6 n*M*. Specific binding was determined by using 3 μ*M* diazepam. Kd = 1.35 n*M*; Bmax = 759.6 fmoles/mg protein

death-to-freezing intervals were not different in the two groups. Of the schizophrenics, 5 patients had been treated with antipsychotics and 3 with BZs until immediately before death. Three patients had received BZs, but not for at least 30 days before death. Brains were stored at −80°C before dissection.

Membrane Preparation. The frozen brains were sectioned as previously described (Toru et al. 1988). In the present study, we examined the medial frontal cortex (areas 9, 10 and 46 according to Brodmann's cytoarchitectonic map), orbitofrontal cortex (areas 45 and 47), eye movement area (area 8), orbital cortex (areas 11 and 12), motor cortex (area 4), superior temporal gyrus (areas 22, 38, 41, 42 and 52), medial and inferior temporal gyri (areas 20 and 21), lateral occipitotemporal gyrus (area 36), secondary and tertiary visual cortex (visual area II, III, areas 18 and 19), dentate gyrus, cornu Ammonis (CA) 1–3 and putamen. These brain tissues were homogenized with a glass-Teflon homogenizer in 4 vol of chilled 0.32 *M* sucrose and stored at −80°C until use.

On the day of assay, the homogenates were thawed and diluted to 25 vol of the original tissue by 50 m*M* TRIS-HCl buffer (pH 7.4) and centrifuged for 15 min at 39,000 *g* at 4°C. The pellets were suspended in 25 vol of the buffer and recentrifuged. The final pellet from the third centrifugation was resuspended in 160 vol of the buffer.

Radioreceptor Assay. In an ice bath, 0.4 ml of suspension containing 0.2 mg protein and 0.8 ml of TRIS-HCl buffer (pH 7.4) containing [^{3}H]-flunitrazepam (85 Ci/mmol, Amersham, U.K.) to give a final concentration of 1 n*M* were incubated in triplicate for 60 min. Specific binding was determined as the difference between total binding and binding in the presence of 3 μ*M* unlabelled diazepam (Sumitomo, Japan). The mixture was filtered by a cell harvester (Brandel, USA) through a Whatman GF/B filter, which was washed three times with 4 ml of ice-cold TRIS-HCl buffer (pH 7.4). The filters were extracted in 10 ml of scintillation cocktail (containing 7% H_2O) and after keeping 4°C overnight, shaken for 15 min and counted for tritium. Protein was assayed by the method of Lowry et al. (1951).

Preliminary studies indicated that unlabelled clonazepam (Sumitomo, Japan), lorazepam (Yamanouchi, Japan), FNT (Roche, Japan) and diazepam displaced [^{3}H]-FNT binding with IC_{50} of 1.6, 4.1, 6.5, and 33.2 n*M* respectively, when measured in the presence of 1 n*M* [^{3}H]-FNT in a membrane preparation from the medial and inferior temporal gyri of a control patient (Fig. 1). All BZs used gave specific binding of 92%–93% of the total at a concentration of 10 μ*M*. Therefore, we used 3 μ*M* diazepam for determining specific binding. It was also indicated, by Scatchard analysis using [^{3}H]-FNT in the concentrations from 0.1–6 n*M* in the same membrane preparation (Fig. 2), that there was only one binding site with high affinity (Kd = 1.35 n*M*).

Specific binding to type 1 and type 2 BZ binding sites in the superior temporal gyrus was determined by a modified version of the mathematical method of Niehoff and Whitehouse (1983) using CL 218,872 (American Cyanamid), which binds preferentially to type 1 binding sites. In brief, CL 218,872 in a final concentration of 200 n*M* in the TRIS-HCl buffer was added to the incubation mixture containing membrane and 1 n*M* [^{3}H]-FNT. This mixture was incubated in an ice bath for 60 min. Total binding for [^{3}H]-FNT was determined by the same method as mentioned above using 3 μ*M* diazepam to obtain nonspecific binding. The results obtained were analyzed by the use of the following simultaneous equations: (1) type 1 binding +

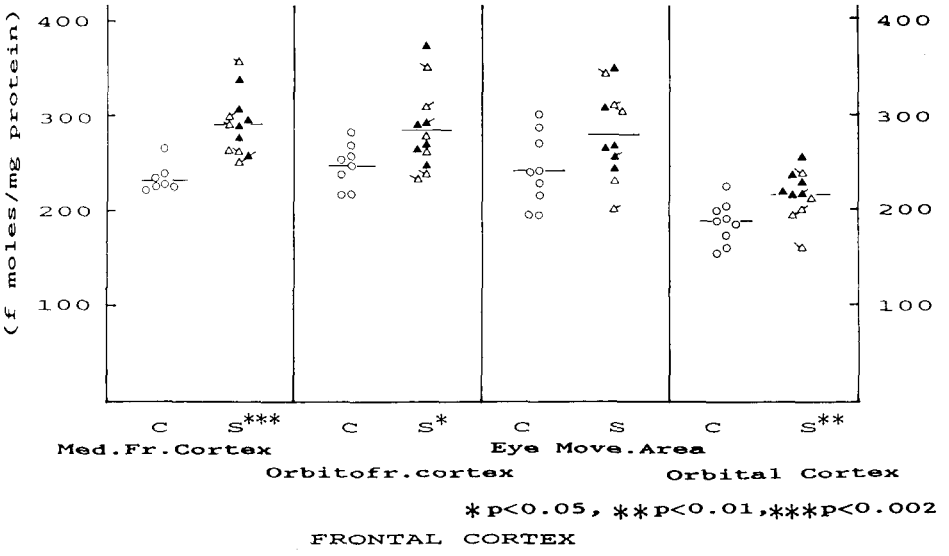

Fig. 3. Specific [^{3}]-FNT binding in the medial frontal cortex, orbitofrontal cortex, eye movement area and orbital cortex of schizophrenics *(S)* and controls *(C)*. (△) "Off-drug" cases, who had received no antipsychotics for more than 30 days before death; (▲) "on-drug" cases, who had been treated with antipsychotics until immediately before death. (△ or ▲) Cases treated with BZs until immediately before death; (△) cases treated with BZs, but not for at least 30 days before death. *Horizontal bars* indicate mean values. In the medial frontal cortex, a significant increase was found in "off-drug" cases compared to controls ($P < 0.01$)

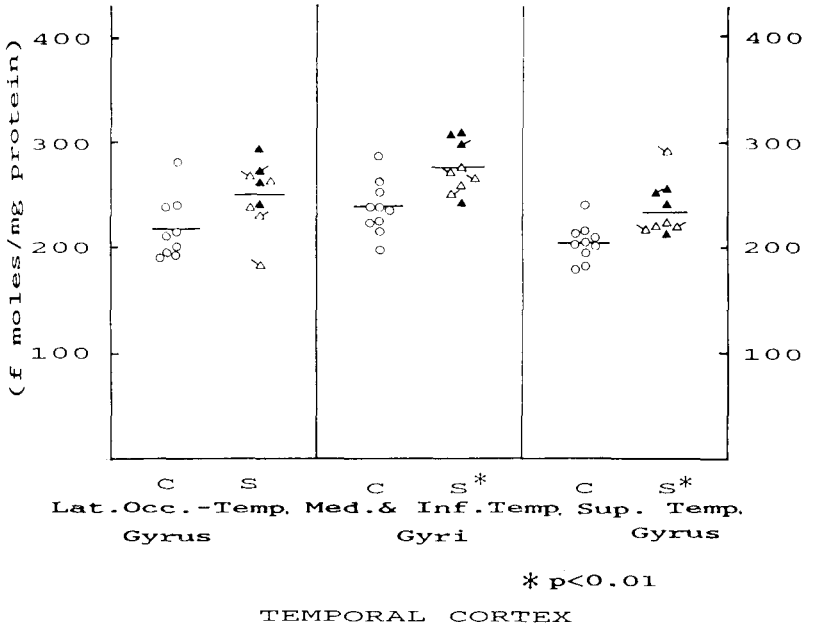

Fig. 4. Specific [^{3}H]-FNT binding in the lateral occipitotemporal gyrus, medial and inferior temporal gyri and superior temporal gyrus of schizophrenics *(S)* and controls *(C)*. In the medial and inferior temporal gyri and superior temporal gyrus, the specific binding was significantly greater in "off-drug" cases than in controls ($P < 0.05$ and $P < 0.02$, respectively)

type 2 binding = total binding; (2) 0.84 type 1 binding + 0.10 type 2 binding = binding displaced by 200 n*M* CL 218,872.

Statistical Analysis. Mean values of specific [^{3}H]-FNT binding between controls and schizophrenics were compared by the Mann-Whitney *U*-test. The Spearman rank test was used to determine correlations between specific [^{3}H]-FNT binding and: (1) age; (2) concentration of GABA; (3) concentration of glutamic acid; (4) specific [^{3}H]-kainic acid binding; (5) activity of tyrosine hydroxylase; (6) substance *P*-like immunoreactivity in various brain areas; (7) the B_{max} of dopamine D_2 receptors in the putamen determined by [^{3}H]-spiperone. All of these have been previously reported using the same brains (Toru et al. 1982, 1988; Nishikawa et al. 1983).

Results

Specific [^{3}H]-FNT binding

Cerebral Cortex. Figure 3 shows specific [^{3}H]-FNT binding in four areas of the prefrontal cortex. In the

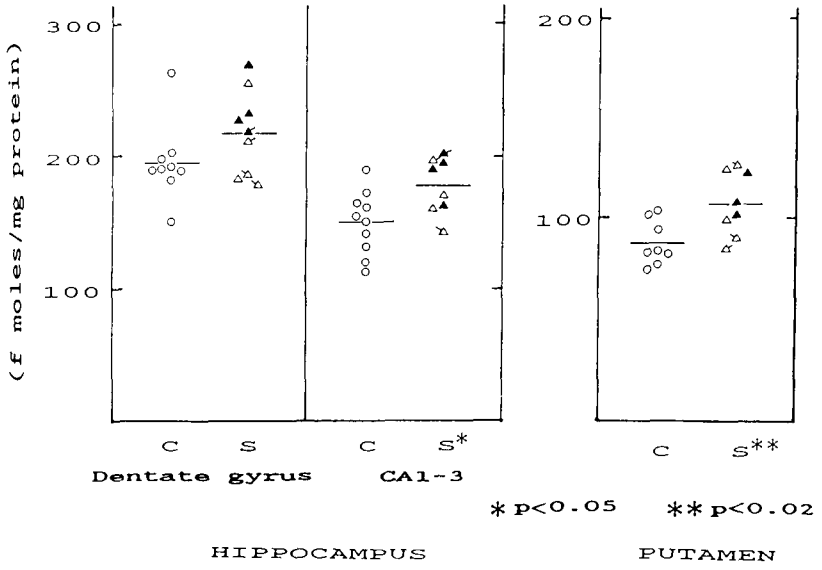

Fig. 5. Specific [^{3}H]-FNT binding in the dentate gyrus, cornu Ammonis 1–3 and putamen of schizophrenics *(S)* and controls *(C)*

medial frontal cortex, specific binding in schizophrenics was 1.25 times that in controls, and this difference was significant ($P < 0.002$). Moreover, specific binding in both "on-drug" patients who had been treated with antipsychotics until immediately before death and "off-drug" patients who had received no antipsychotics for at least 30 days before death was greater than that in controls ($P < 0.002$ and $P < 0.01$, respectively). Specific binding in the orbitofrontal and orbital cortices was also greater in schizophrenics ($P < 0.05$ and $P < 0.01$, respectively). However, treatment with BZs seemed unlikely to affect specific binding in the brain areas shown in Fig. 3. In the motor cortex, there was no significant difference between the two groups (data not shown).

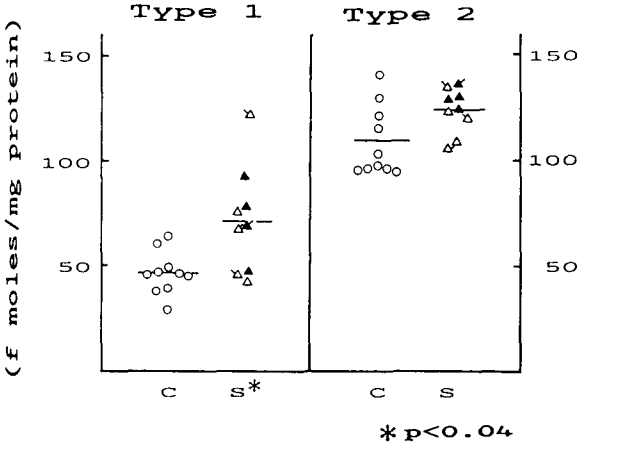

Fig. 6. Specific [^{3}H]-FNT binding to type 1 and type 2 binding sites in the superior temporal gyrus of schizophrenics *(S)* and controls *(C)* determined by a mathematical method using 200 n*M* CL 218,872

In the case of the three areas in the temporal cortex (Fig. 4), specific [^{3}H]-FNT binding in the medial and inferior temporal gyri and superior temporal gyrus was significantly greater in schizophrenics than in controls ($P < 0.01$). Specific binding in the above two areas of "off-drug" patients was also significantly greater than that of controls ($P < 0.05$ and $P < 0.02$, respectively). No significant difference between the two groups was found in specific binding in the visual area II, III (data not shown).

Hippocampus and Putamen. Specific binding in the cornu Ammonis 1–3 but not in the dentate gyrus was significantly greater in schizophrenics than in controls ($P < 0.05$). The mean value of specific binding in the cornu Ammonis 1–3 was about two-thirds that in the cerebral cortex (Fig. 5).

Specific binding in the putamen of schizophrenics (Fig. 5), which was about 40% of that in the cortex, was significantly greater than that in controls ($P < 0.02$).

Subtypes of BZ Receptors in Superior Temporal Gyrus

Figure 6 shows type 1 and type 2 BZ binding values of [^{3}H]-FNT in the superior temporal gyrus, determined by 200 n*M* CL 218,872. In controls, the proportion of type 1 binding sites was calculated to be 30% of the total specific binding sites for [^{3}H]-FNT. Type 1 BZ binding in schizophrenics was significantly greater than that in controls (+ 54%, $P < 0.04$). There was an increased tendency for type 2 BZ binding in comparison with controls (+ 13%), but the difference was not significant. Consequently, type 1 BZ binding sites occupied 36% of the total specific binding sites of [^{3}H]-FNT in schizophrenics.

Discussion

In this study, there was a negative correlation between age and specific [^{3}H]-FNT binding in 8 of 12 brain areas when calculated for both control and schizophrenic groups combined. No significant correlation was found between age and specific [^{3}H]-FNT binding in controls, whereas significant negative correlations were found in the schizophrenic group in three brain areas: eye movement area ($r = -0.69$, $P < 0.05$); orbitofrontal area ($r = -0.85$, $P < 0.001$); secondary and tertiary visual cortex ($r = -0.66$, $P < 0.05$). The effects of age on BZ receptor characteristics in animal studies are conflicting, but most studies show little effect of aging on binding (Tsang et al. 1982; Miller et al. 1987; Niles et al. 1988). The effect of aging on BZ receptors in human brain has not yet been sufficiently studied. Although there was no significant difference between the ages of schizophrenics and controls in the present study, the age of controls tended to be higher than that of schizophrenics. Specific [^{3}H]-FNT binding in controls was lower than in schizophrenics. These two factors might be the cause of the apparent negative correlation of binding values with age in many brains areas.

There have been few reports of the effects of antipsychotic treatment on BZ receptor binding. Rupniak et al. (1987) observed about 30% decrease in the B_{max} of [^{3}H]-FNT binding in rat cerebellum, but not in the striatum after treatment for 12 months with haloperidol, sulpiride and clozapine. The Kd values in both areas were unchanged. In our study, specific [^{3}H]-FNT binding of "off-drug" cases was significantly higher than those of controls in three brain areas. Thus, it seems that antipsychotic treatment did not greatly affect the increase in specific [^{3}H]-FNT binding in schizophrenics.

There are conflicting reports about the changes in the numbers or characteristics of BZ receptor binding sites in the brain after chronic BZ administration. Several investigators have reported a decrease in [^{3}H]-FNT or [^{3}H]-diazepam binding after chronic BZ treatment of rats (Crawley et al. 1982; Tietz et al. 1986), whereas other groups have observed no change (Möhler et al. 1978) or increase (DiStefano et al. 1979). Thus, the effects of chronic BZ treatment on the receptor binding remain uncertain. Some of these discrepancies seem to be attributable to the variety of treatment conditions in these experiments. As shown in Figs. 3–5, there was no clear difference between specific [^{3}H]-FNT binding in patients who had been treated with BZs before death and those who had not.

Scatchard analysis using membrane preparation of the medial and inferior temporal gyri indicated that the high values in schizophrenics seemed to be due to an increase in the B_{max} of [^{3}H]-FNT binding, not to a change in the affinity (data not shown). Moreover, we observed a significant increase (54%) in type 1 and a non-significant increase (13%) in type 2 binding sites in the superior temporal gyrus of schizophrenics determined from displacement of [^{3}H]-FNT binding by CL 218,872. Since [^{3}H]-FNT binds to both types of binding sites with the same affinity, this result suggests that increased specific [^{3}H]-FNT binding was due more to the increase in type 1 binding sites than in type 2 binding sites.

The heterogeneous distribution of BZ receptor subtypes has been reported in rat (Braestrup and Nielsen 1981; Young et al. 1981) and man (Corda et al. 1988). The cerebellum contained almost exclusively type 1 BZ binding sites, whereas the cerebral cortex and hippocampus contained both subtypes of BZ receptors. In the present study, the proportions of types 1 and 2 BZ binding sites in the superior temporal gyrus were 30/70 in controls and 36/64 in schizophrenics.

At present, the pharmacological and clinical characteristics of subtypes of BZ receptors are not fully evaluated. CL 218,872 was initially reported to possess anti-conflict and anti-convulsant properties but to be devoid of sedative activity (Lippa et al. 1979), but later studies have described a reduction in motor activity (Oakley et al. 1984) and a prolongation of sleep time (Mendelson et al. 1988) by this ligand in rats. Therefore, it seems difficult to conclude that there is any functional significance from our results, which show a significant increase in type 1 BZ binding sites in chronic schizophrenics, because the functions mediated by each subtype of BZ receptors are controversial.

There have been no previous data on binding studies of BZ receptors in post-mortem brains of schizophrenics. However, increasing evidence points to the involvement of an alternation in BZ receptor function in some psychiatric conditions. Manchon et al. (1987) studied the characteristics of BZ receptors on the hippocampus of suicides. They reported an increase in the Ki of CL 218.872 for [^{3}H]-FNT binding and an increase in the proportion of type 1 binding sites in suicides. Barbaccia et al. (1986) observed that patients with major depression had significantly higher concentration of diazepam-binding inhibitor (DBI)-like immunoreactivity in CSF, while no significant differences were found between schizophrenics or patients with Alzheimer's disease and controls.

To obtain a better understanding of changes in the BZ receptors in schizophrenics, we analyzed the correlations between specific [^{3}H]-FNT binding and the other biochemical data that had been obtained previously using the same brain samples. Specific [^{3}H]-FNT

binding in many brain areas correlated positively with GABA concentration in four brain areas, specific [^{3}H]-kainic acid binding in eight brain areas, activity of tyrosine hydroxylase in six brain areas, and substance P-like immunoreactivity in six brain areas, and with the B_{max} of [^{3}H]-spiperone binding in the putamen. Negative correlations were found between [^{3}H]-FNT binding and glutamic acid concentrations in three brain areas. We found significant changes in all of these substrates and receptors except for GABA and glutamic acid concentrations in several brain areas of schizophrenics compared with controls, and we have also reported correlations between these neurotransmitters, receptors and neuropeptides (Toru et al. 1988). It may thus be assumed from our data that many biochemical changes coexist in the brain of chronic schizophrenics. Moreover, we have speculated that dopaminergic hyperactivity is not only important in acute psychotic symptoms in schizophrenics, but also continues to exist in chronic patients, and that these biochemical changes observed in chronic schizophrenia might be a result of the hyperdopaminergic state (Toru et al. 1988).

There have been several observations about the effects of BZs on dopaminergic system. Diazepam and chlordiazepoxide have been reported to reduce dopamine metabolism in the striatum, tuberculum olfactorium and nucleus accumbens (Fuxe et al. 1975; Singhal et al. 1983). It has been speculated that enhancement of $GABA_A$ receptor activity, which exerts an inhibitory influence on dopaminergic neurotransmission, could be mainly involved in causing reduction of dopamine turnover observed after BZs (Fuxe et al. 1975). Therefore, the increased [^{3}H]-FNT binding in chronic schizophrenics may be associated with the enhancement of dopaminergic transmission occurring in this disease. The finding of good positive correlations between the [^{3}H]-FNT binding and activity of tyrosine hydroxylase or between [^{3}H]-FNT binding and D_2 receptor binding might agree with this interpretation.

Finally, we would like to discuss the clinical significance of our findings. DBI and β-carbolines have been shown to be proconflictive and proconvulsive and to induce an increase in muscle tension and the level of arousal (Guidotti et al. 1983; Skolnick and Paul 1983; Insel et al. 1984; Dorow et al. 1987). If endogenous ligands have characteristics like those "inverse agonists," anxiety and hyperarousal, which are most frequently observed in schizophrenics, might be based on the increase of BZ receptors in these patients. The increase in muscle tone observed in catatonics might also be associated with this alternation. Parts of the limbic system, such as the hippocampus and amygdala, are assumed to be involved in anxiety (Braestrup and Nielsen 1982), and the putamen is relevant to the regulation of muscle tone. Therefore, it should be noted that increase in specific [^{3}H]-FNT binding was observed not only in cortical regions, which are supposed to be important regions involved in the major psychotic symptoms of schizophrenics, but also in the cornu Ammonis 1–3 and putamen. Thus, it is possible that an alternation in BZ receptors might be partially associated with clinical symptoms of chronic schizophrenia. Several psychiatrists have used BZs in managing schizophrenic symptoms, with various results (Arana et al. 1986). Lerner et al. (1979) found that parenteral diazepam was equally as effective as parenteral haloperidol for the treatment of psychotic symptoms in a double-blind trial. These authors (Lerner et al. 1979; Arana et al. 1986) suggested that some schizophrenic symptoms may be secondary to arousal or anxiety. It is still unknown whether BZs and inverse agonists act on the same receptor sites in the same manner. Thus, new drugs that antagonize the actions of endogenous ligands for BZ receptors in the human brain will be needed to prove the role of the present findings in the pathogenesis of schizophrenic symptoms.

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