

## Effects of *Kamikihito*, a Traditional Chinese Medicine, on Neurotransmitter Receptor Binding in the Aged Rat Brain Determined by In Vitro Autoradiography (2): Changes in GABA<sub>A</sub> and Benzodiazepine Receptor Binding

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**ABSTRACT**—We investigated the effects of the long-term administration of *Kamikihito* (KKT) on the specific binding of [<sup>3</sup>H]muscimol and [<sup>3</sup>H]flunitrazepam in the brains of young and aged rats using in vitro quantitative autoradiography. Specific [<sup>3</sup>H]muscimol binding in aged rats was decreased in all brain regions examined compared with that in young rats, whereas [<sup>3</sup>H]flunitrazepam binding did not change in any of the brain regions. Scatchard analysis revealed that the maximal number of [<sup>3</sup>H]muscimol binding sites in the cortex and thalamus was significantly decreased in aged rats compared with young rats, while its affinity remained unchanged. Long-term administration of KKT in young rats had no effect on either [<sup>3</sup>H]muscimol or [<sup>3</sup>H]flunitrazepam binding. In contrast, the same treatment in aged rats produced a significant increase in [<sup>3</sup>H]flunitrazepam binding to the cortex, caudate/putamen and accumbens, and it tended to decrease the [<sup>3</sup>H]muscimol binding. These results suggest that the selective reduction of specific [<sup>3</sup>H]muscimol binding in the brain may be responsible, at least in part, for anxiety-related behavior in aged rats. Furthermore, it appears that the significant increase in specific [<sup>3</sup>H]flunitrazepam binding produced in the brains of aged rats by the long-term administration of KKT may be responsible for the anxiolytic effects of this agent.

**Keywords:** *Kamikihito*, Aged rat, [<sup>3</sup>H]Muscimol binding, [<sup>3</sup>H]Flunitrazepam binding, Autoradiography

*Kamikihito* (KKT), which consists of Astragalus, Ginseng, Atractylodes, Hoelen, Polygala, Jujube, Longan, Zizyphus, Angelica, Licorice, Ginger, Saussurea, Bupleurum and Gardenia, is a traditional Chinese medicine that is used to treat anemia, insomnia, anxiety and neurosis. In addition to exerting an anxiolytic effect, it has been reported that KKT improved the cognitive ability of alcoholic patients (1). Furthermore, it has been shown that KKT improves learning performance in the senescent accelerated mouse (2). These findings suggest that KKT may ameliorate anxiety, as well as memory impairment, in the elderly. Although we have recently demonstrated that long-term administration of KKT modulates the binding of [<sup>3</sup>H]quinuclidinyl benzilate (QNB) and [<sup>3</sup>H]N-(1-[2-thienyl]cyclohexyl)-3,4-piperidine (TCP) to rat brain

slices by in vitro quantitative autoradiography (3), the mechanisms underlying the anxiolytic and anti-amnesic effects of KKT are largely unknown.

In previous studies (4–6), we found that, in aged rats, not only were learning and memory impaired, but emotional behavior was also altered. Since psychotic symptoms, including anxiety, depression, delusions and hallucinations, are a frequent accompaniment of memory impairment in the elderly (7, 8), our findings in the earlier studies suggest that aged rats are useful for investigating the mechanisms underlying the aging-associated decline of brain function, as well as for predicting the clinical effects of drugs in aged patients.

In the present study, using in vitro quantitative autoradiography, we compared the specific binding of [<sup>3</sup>H]muscimol and [<sup>3</sup>H]flunitrazepam in the brains of aged rats with that in young rats, since these two neurotransmit-

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ter receptors are very important in anxiety. We also investigated the effects of long-term administration of KKT on the binding of these receptors.

## MATERIALS AND METHODS

### Materials

We used aged male Fischer rats (99-week-old before the experiment; Charles River Japan, Inc, Hino, Shiga).

Young control rats were 6-week-old before the experiment. All animals were kept in a temperature- and light-controlled room (23°C, 12-hr light cycle starting at 9:00 a.m.). They were given a regular diet or one containing KKT (8%; Kanebo Co., Ltd., Tokyo) for 15 weeks. They were then sacrificed for autoradiography, at which time the aged and young rats were 114- and 21-week-old, respectively. The calculated daily doses of KKT in young and aged rats were 1.25 and 1.36 g/rat, respectively. The body weights of aged rats given the regular and KKT-containing diets after the 15-week period of drug administration were  $484 \pm 4$  and  $461 \pm 5$  g, respectively, while those of young rats given the regular and KKT-containing diets were  $287 \pm 17$  and  $278 \pm 15$  g, respectively. [ $^3\text{H}$ ]Muscimol (specific activity, 20.0 Ci/mmol) and [ $^3\text{H}$ ]flunitrazepam (specific activity, 74.6 Ci/mmol) were acquired from NEN Research Products (Boston, MA, USA). [ $^3\text{H}$ ]Microscale (tissue equivalent values, 0.072–32.0 nCi/mg) was obtained from Amersham (Amersham, Buckinghamshire, UK). GABA was purchased from Sigma (St. Louis, MO, USA). Chlordiazepoxide was kindly provided by Hoffman-La Roche (Basel, Switzerland).

### Tissue preparation and in vitro autoradiography

Rats were sacrificed by decapitation, and their brains were removed and rapidly frozen at  $-100^\circ\text{C}$ . Twenty-micron cryostat sections were prepared for the binding assay. Autoradiography of [ $^3\text{H}$ ]muscimol (2–64 nM) and [ $^3\text{H}$ ]flunitrazepam (1 nM) was carried out as described previously (9, 10). Briefly, sections on the slide glass were incubated with each [ $^3\text{H}$ ]ligand at  $4^\circ\text{C}$  for 40 min. After incubation, they were rinsed 3 times in ice-cold 170 mM Tris-HCl buffer (pH 7.4) at  $4^\circ\text{C}$  for 10 sec each time, followed by one rinse in ice-cold distilled water; they were then dried in a stream of cold air. Non-specific binding of [ $^3\text{H}$ ]muscimol and [ $^3\text{H}$ ]flunitrazepam was defined as binding in the presence of 1 mM GABA and 1 mM chlordiazepoxide, respectively. After the binding assay, the slides were tightly apposed to hyperfilm [ $^3\text{H}$ ] with a [ $^3\text{H}$ ]microscale and stored at  $4^\circ\text{C}$  for 15 to 21 days. After exposure, the film was developed in D-19 (Kodak, Tokyo) at  $20^\circ\text{C}$  for a few minutes. The autoradiogram was placed in a photographic illumination apparatus (Northern Light; Imaging Research Inc., St. Catharines, Canada), and the

optical density of the various regions of interest was measured with an image analyzer (MCID system, Imaging Research, Inc.). The amount of [ $^3\text{H}$ ]ligand was determined by comparing autoradiograms of the [ $^3\text{H}$ ]microscale; the specific binding was calculated by subtracting the non-specific binding from the total binding, by using a computer system. The specific binding of [ $^3\text{H}$ ]muscimol and [ $^3\text{H}$ ]flunitrazepam represented approximately 95% and 85% of the total binding, respectively. The  $B_{\text{max}}$  and  $K_d$  values for [ $^3\text{H}$ ]muscimol binding were calculated by the BMDP iterative non-linear program AR (11).

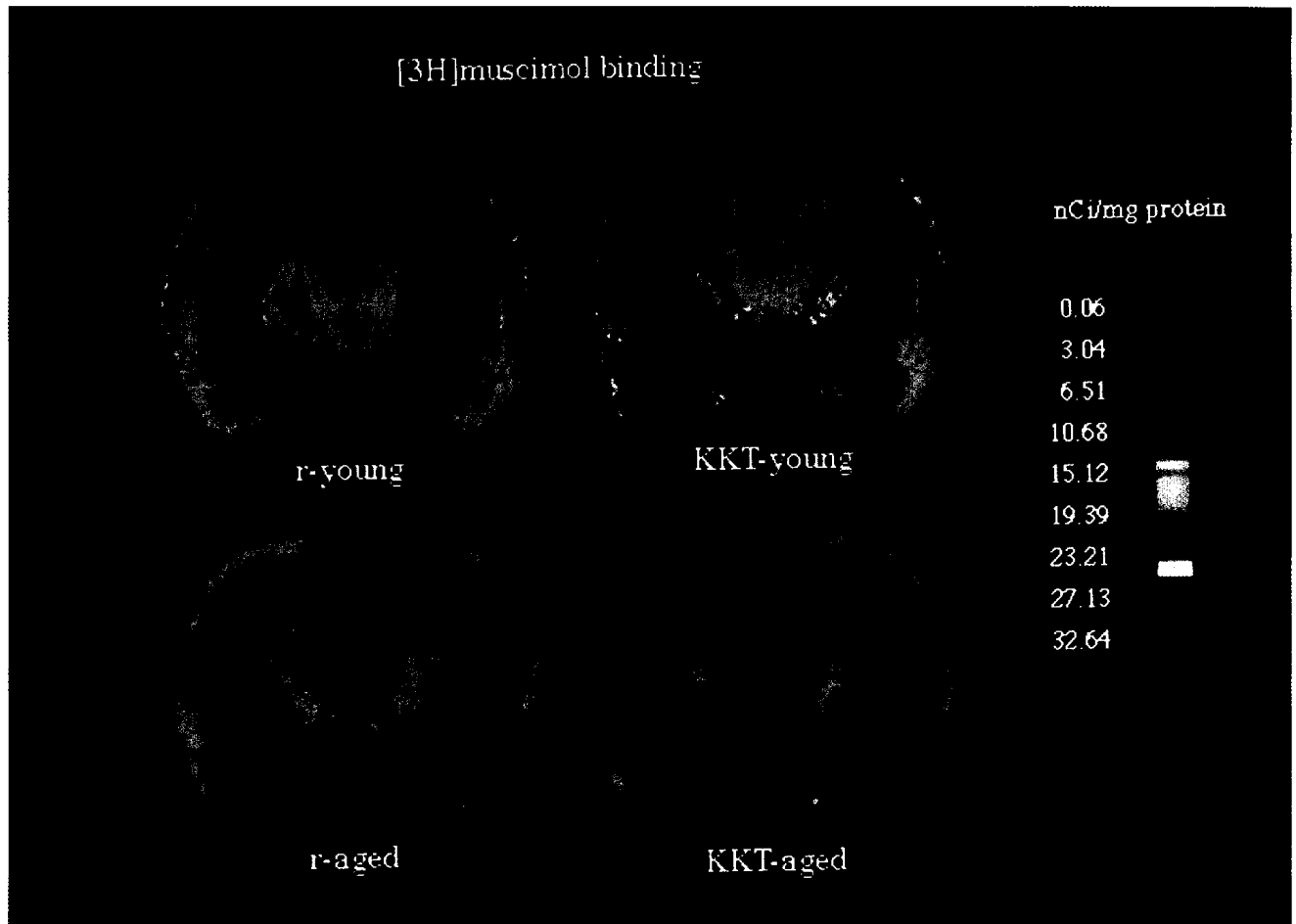
### Statistical analyses

Results are expressed as means  $\pm$  S.E. ( $n=5$ ). The significance of differences was assessed by the two-tailed Student's *t*-test.

## RESULTS

The representative color-imaged autoradiograms of the specific binding of [ $^3\text{H}$ ]muscimol and [ $^3\text{H}$ ]flunitrazepam are shown in Figs. 1 and 2, respectively, in coronal sections of the brains in young and aged rats given regular or KKT-containing diet. The specific binding of [ $^3\text{H}$ ]muscimol (64 nM) in aged rats was decreased in all brain regions examined compared with that in young rats (Table 1). Scatchard analysis revealed that the maximal number of binding sites ( $B_{\text{max}}$ ) of [ $^3\text{H}$ ]muscimol in the cortex and thalamus were significantly decreased in aged rats compared with young rats, while the  $K_d$  value remained unchanged (Table 2). There was a similar reduction of the  $B_{\text{max}}$  but not  $K_d$  values for [ $^3\text{H}$ ]muscimol binding in aged rats compared with young rats, although the differences were not statistically significant: The  $B_{\text{max}}$  of [ $^3\text{H}$ ]muscimol in the caudate/putamen, accumbens, hippocampus and amygdala in aged rats were decreased to 86, 57, 75 and 80% of those in young rats, respectively (data not shown). There were no differences between young and aged rats in the specific binding of [ $^3\text{H}$ ]flunitrazepam (1 nM) to any of the examined brain regions (Table 3).

Long-term administration of KKT in young rats had no effect on either [ $^3\text{H}$ ]muscimol (Table 1) or [ $^3\text{H}$ ]flunitrazepam binding (Table 3). In contrast, the same treatment in aged rats tended to decrease the [ $^3\text{H}$ ]muscimol binding (Table 1). Furthermore, there was a significant increase in the specific binding of [ $^3\text{H}$ ]flunitrazepam in the cortex, caudate/putamen and accumbens of aged rats given the KKT-containing diet (KKT-aged rats) compared with that in aged rats given the regular diet (r-aged rats) (Table 3).



**Fig. 1.** Color-imaged autoradiograms of the specific [<sup>3</sup>H]muscimol (64 nM) binding in the coronal sections of the brain in young and aged rats. The autoradiogram of the [<sup>3</sup>H]micro-scale, which is used for quantitative analysis, shows the standard tritium concentration sequences. r-young: young rats given regular diet, KKT-young: young rats given KKT-containing diet, r-aged: aged rats given regular diet, KKT-aged: aged rats given KKT-containing diet.

**Table 1.** Binding of [<sup>3</sup>H]muscimol in the brains of young and aged rats given a regular or KKT (8%)-containing diet for 15 weeks

Brain region	r-young	KKT-young	r-aged	KKT-aged
Cortex	936 ± 29	965 ± 22	717 ± 37***	641 ± 20
Caudate/Putamen	415 ± 24	420 ± 26	303 ± 26**	269 ± 30
Accumbens	487 ± 15	485 ± 23	317 ± 26***	289 ± 36
Hippocampus	667 ± 29	683 ± 23	548 ± 30*	468 ± 31
Thalamus	831 ± 41	745 ± 56	626 ± 59*	556 ± 24
Amygdala	605 ± 31	562 ± 42	482 ± 18**	409 ± 26

Specific [<sup>3</sup>H]muscimol (64 nM) binding is expressed as fmol/mg protein. r-young: young rats given regular diet, KKT-young: young rats given KKT-containing diet, r-aged: aged rats given regular diet, KKT-aged: aged rats given KKT-containing diet. Each value represents a mean ± S.E. (n = 5). \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 vs. r-young rats.

**Table 2.** Scatchard analysis of [<sup>3</sup>H]muscimol binding in different brain regions of young and aged rats

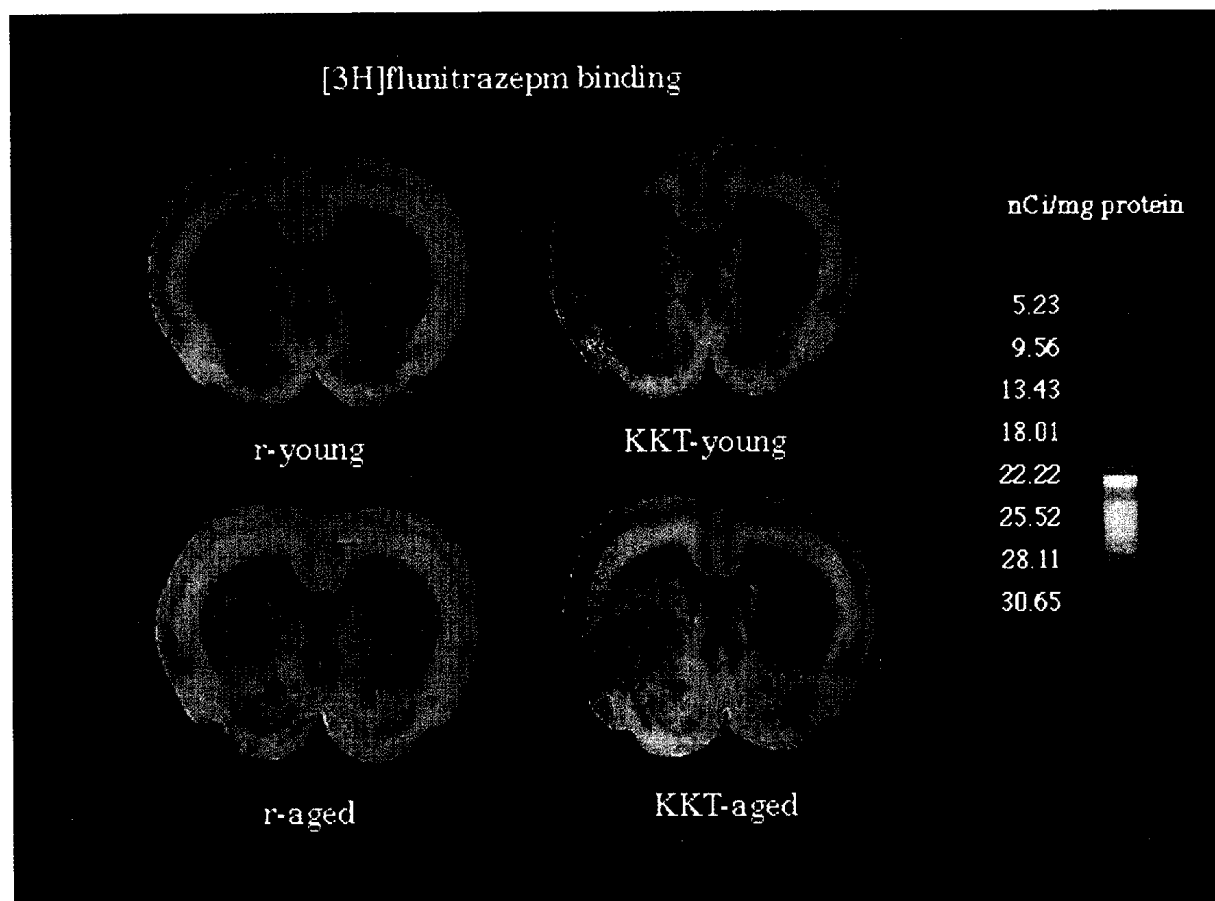
Brain region	B <sub>max</sub> (pmol/mg protein)		K <sub>d</sub> (nM)	
	young	aged	young	aged
Cortex	1.229 ± 0.068	0.987 ± 0.055*	16.3 ± 2.4	19.2 ± 4.8
Thalamus	1.175 ± 0.067	0.930 ± 0.040*	21.7 ± 1.1	22.8 ± 3.2

Each value represents a mean ± S.E. (n = 5). \*P < 0.05 vs. young rats.

## DISCUSSION

### *Changes in [<sup>3</sup>H]muscimol and [<sup>3</sup>H]flunitrazepam binding in aged rats*

Using in vitro autoradiography, we compared the specific binding of [<sup>3</sup>H]muscimol and [<sup>3</sup>H]flunitrazepam



**Fig. 2.** Color-imaged autoradiograms of the specific [ $^3\text{H}$ ]flunitrazepam (1 nM) binding in the coronal sections of the brain in young and aged rats. The autoradiogram of the [ $^3\text{H}$ ]micro-scale, which is used for quantitative analysis, shows the standard tritium concentration sequences. r-young: young rats given regular diet, KKT-young: young rats given KKT-containing diet, r-aged: aged rats given regular diet, KKT-aged: aged rats given KKT-containing diet.

**Table 3.** Binding of [ $^3\text{H}$ ]flunitrazepam in the brains of young and aged rats given a regular or KKT (8%)-containing diet for 15 weeks

Brain region	r-young	KKT-young	r-aged	KKT-aged
Cortex	351 $\pm$ 6	356 $\pm$ 10	341 $\pm$ 3	356 $\pm$ 4**
Caudate/Putamen	238 $\pm$ 7	250 $\pm$ 13	239 $\pm$ 4	257 $\pm$ 7*
Accumbens	271 $\pm$ 6	286 $\pm$ 13	283 $\pm$ 9	302 $\pm$ 5*
Hippocampus	344 $\pm$ 9	339 $\pm$ 23	327 $\pm$ 6	332 $\pm$ 5
Thalamus	218 $\pm$ 8	213 $\pm$ 12	206 $\pm$ 6	217 $\pm$ 7
Amygdala	342 $\pm$ 4	348 $\pm$ 7	331 $\pm$ 7	346 $\pm$ 7

Specific [ $^3\text{H}$ ]flunitrazepam (1 nM) binding is expressed as fmol/mg protein. r-young: young rats given regular diet, KKT-young: young rats given KKT-containing diet, r-aged: aged rats given regular diet, KKT-aged: aged rats given KKT-containing diet. Each value represents a mean  $\pm$  S.E. (n = 5). \*P < 0.05 and \*\*P < 0.01 vs. r-aged rats.

in the brains of aged rats with that in young rats. Previous studies have provided somewhat controversial results, depending on the brain region and animal species used (12–14). For example, Ito et al. (13) have reported that [ $^3\text{H}$ ]muscimol binding increased in aged mice, while others observed no change in this binding in aged rats (12, 14). We demonstrated here, using in vitro quantitative autoradiography, that the specific binding of [ $^3\text{H}$ ]muscimol in aged rats was decreased in all brain regions examined, compared with that in young rats, whereas [ $^3\text{H}$ ]flunitrazepam binding did not change in any brain region. The ratios of [ $^3\text{H}$ ]muscimol and [ $^3\text{H}$ ]flunitrazepam binding were significantly different between young and aged rats: those in the young rats were  $2.67 \pm 1.0$ ,  $1.74 \pm 0.07$ ,  $1.94 \pm 0.06$  and  $3.81 \pm 0.10$  in the cortex, caudate/putamen, hippocampus and thalamus, respectively, while those in the aged rats were  $2.10 \pm 0.09$ ,  $1.27 \pm 0.11$ ,  $1.67 \pm 0.07$  and  $3.04 \pm 0.27$ , respectively. Scatchard analysis revealed that the reduction in [ $^3\text{H}$ ]muscimol binding to

the cortex and thalamus in aged rats was solely due to a decrease in the  $B_{\max}$ , but not the  $K_d$ , of this binding. Since GABA exerts its effects, in part, by interacting with the GABA<sub>A</sub>/benzodiazepine/ $\text{Cl}^-$  channel complex, our findings suggest that changes in allosteric interaction between GABA<sub>A</sub> and benzodiazepine receptors may occur in the brains of aged rats.

The GABA<sub>A</sub> receptor is a hetero-oligomeric protein composed of several distinct polypeptide types (15). It has been demonstrated that the mRNA for the  $\alpha_1$ -subunit of the GABA<sub>A</sub> receptor in the cerebral cortex and the  $\alpha_2$  mRNA in the cerebellum are downregulated, and that  $\alpha_6$  mRNA levels in the cerebellum are increased by aging in Fischer F-344 rats (16). Taken together, the observed changes in the binding in aged rats may be due to the altered regulation of gene transcription of GABA<sub>A</sub> receptor subunits.

The changes in [<sup>3</sup>H]muscimol and [<sup>3</sup>H]flunitrazepam binding in the brain could be responsible, at least in part, for brain dysfunction in aged rats, as indicated by the impairment of learning and memory, and by their altered emotional behavior (4–6, 17, 18). Interestingly, we have observed similar changes (reduction of [<sup>3</sup>H]muscimol binding but no change in [<sup>3</sup>H]flunitrazepam binding) in mice that had acquired tolerance to the anxiolytic effects of chlordiazepoxide (9). Therefore, we consider that behavior indicating anxiety in aged rats, which includes decreases in time spent in social interaction between pairs from different cages and increases in starting latency, defecation and urination in an open field, may be related to a reduced number of GABA<sub>A</sub> receptors in the brain.

#### *Effects of Kamikihito on [<sup>3</sup>H]muscimol and [<sup>3</sup>H]flunitrazepam binding*

Long-term administration of KKT in young rats had no effect on either [<sup>3</sup>H]muscimol or [<sup>3</sup>H]flunitrazepam binding. In contrast, the same treatment in aged rats produced a significant increase in [<sup>3</sup>H]flunitrazepam binding to the cortex, caudate/putamen and accumbens, and tended to decrease the [<sup>3</sup>H]muscimol binding. These results suggest that KKT may have some improving effects on aging-associated anxiety which is associated with a selective reduction of [<sup>3</sup>H]muscimol binding in the brain. It is unlikely that the changes in the binding in aged rats administered with KKT are due to the direct binding of a constituent of KKT, since the brain sections were preincubated to remove substances that had previously been bound to the binding sites before incubating the tissues with the radioligands.

It is well known that the benzodiazepines exert their anxiolytic effects by binding to benzodiazepine receptors, and then potentiating the action of GABA. Thus, the increase in [<sup>3</sup>H]flunitrazepam binding in the KKT-aged rats

suggests that compensation for the reduced [<sup>3</sup>H]muscimol binding in the brains of these animals may have been brought about by the long-term administration of KKT. It is not yet clear why KKT increases [<sup>3</sup>H]flunitrazepam binding in certain brain regions. One possible explanation is that there may be different subtypes of [<sup>3</sup>H]flunitrazepam binding sites, as previously suggested (19, 20), type one being KKT-sensitive and others being KKT-insensitive. To clarify the effects of KKT on the [<sup>3</sup>H]flunitrazepam binding in aged rats, further studies, including the Scatchard analysis of the binding and the measurement of the mRNA levels encoding GABA<sub>A</sub> receptor subunits, should be carried out.

In conclusion, the results of this study suggest that the decrease in [<sup>3</sup>H]muscimol binding and the lack of change in [<sup>3</sup>H]flunitrazepam binding in the brains of aged rats may be responsible, at least in part, for their anxiety-related behavior. The present findings also support previous results showing that KKT has clinical anxiolytic effects, and they suggest that the increase in [<sup>3</sup>H]flunitrazepam binding in the cortex, caudate/putamen and accumbens seen in KKT-treated aged rats may be responsible for the anxiolytic effects of this agent.

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