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An acute effect of triazolam on muscarinic cholinergic receptor binding in the human brain measured by positron emission tomography

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Abstract. An acute effect of triazolam, a potent benzodiazepine agonist, on cholinergic receptor binding in the human brain was measured by PET (positron emission tomography) using [^{11}C]*N*-methyl-4-piperidylbenzilate ([^{11}C]NMPB), a potent muscarinic cholinergic receptor antagonist. Two PET scans were performed in each subject: (1) control scan; (2) after oral administration of 0.5 mg triazolam or placebo. The previously discussed amnestic effect of triazolam was measured by immediate and delayed recall of meaningful and meaningless syllables. A compartment model employing the radioactivity in the cerebellum as an input function was used for the quantification of receptor binding. The binding parameter, k_3, was decreased after triazolam administration in all measured regions, whereas no change was observed after placebo treatment. The reduction compared to the control study varied from $8.6 \pm 3.7\%$ in the temporal cortex to $16.3 \pm 6.3\%$ in the thalamus. Triazolam administration impaired both immediate and delayed recall of syllables, whereas placebo administration had no effects. Benzodiazepine agonists are reported to decrease the cortical acetylcholine release. The decrease of acetylcholine release in the synaptic cleft might be the explanation for the decreased binding of [^{11}C]NMPB.

Key words: Muscarinic acetylcholine receptors – Benzodiazepine – Neural interactions – PET – NMPB – Human brain – Triazolam – Memory – Amnesia

The functions of the brain are the result of numerous neurochemical actions and in the living human brain many neurotransmitter systems are believed to interact with each other. However, most neurochemical experiments have focused on the single neurotransmitter system in vitro, and even in vivo. However, in vivo, significant interactions between the different neural systems can be observed that can not be observed in vitro (Dewey et al. 1990; Suhara et al. 1990). Positron emission tomography (PET) made it possible to measure the neurotransmitter receptors in the living human brain and the interactions of neurotransmitter systems (Dewey et al. 1990).

Benzodiazepines are known to produce anterograde amnesia as a side effect (Curran 1991; King 1992). The underlying mechanism of amnesia caused by benzodiazepines is not clear, but it is well known that the cholinergic neural system plays a very important role in learning and memory (Drachman and Leavitt 1974; Sitaram et al. 1978). Some clinical studies show that diazepam and scopolamine have similar effects on certain memory tests (Ghoneim and Mewaldt 1975; Frith et al. 1984). Animal studies show that the disruptive effects of chlordiazepoxide on learning and memory in mice were partially antagonized by the choline esterase inhibitor (Nabeshima et al. 1990). Some interactions have been reported between the cholinergic system and the GABA-benzodiazepine system (Decker and McGaugh 1991). Diazepam and triazolam increase the concentration of acetylcholine in rat brain (Sethy 1978) due to the reduction of its release (Petkov et al. 1982). It has been suggested that certain benzodiazepines are more selective in interfering with memory (Scharf et al. 1988). Triazolam has been reported to change learning and memory at therapeutic doses (Morris and Estes 1987; Weingartner et al. 1992).

Triazolam is known to be a short-acting hypnotic drug in the usual dosage of 0.25–0.5 mg and it is also known as a potent benzodiazepine agonist with high affinity for benzodiazepine receptors. The peak plasma concentration of triazolam is reached at 1.3 h after oral ingestion and the plasma elimination half-life is about 2.2 h (Pakes et al. 1981).

In the present study, we investigated the acute effect of triazolam on muscarinic cholinergic receptor binding in the human brain, measured by PET, and on memory, measured by immediate and delayed recall of syllables to elucidate the interactions between the GABA-benzodiazepine system and the muscarinic cholinergic system, both of which are closely related to human memory.

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Materials and methods

Subjects. Nine healthy male volunteers (age: 18–24 years; mean 21.3±2.3), with no history of drug- or alcohol-related problems, were recruited primarily from a university community. None of the participants had taken any medicine for several months before the study. They were told to avoid alcohol for at least 24 h. The research project was described to the subjects, and informed consent was obtained prior to participation. All procedures were approved by the Ethics and Radiation Safety Committee of the National Institute of Radiological Sciences, Chiba.

Study design. Two PET scans were performed in each subject: (1) control scan; (2) after oral administration of 0.5 mg triazolam or placebo. The two experiments were performed on the same day for seven subjects (four triazolam, three placebo). The control experiment started about 10 a.m. after a 20-min transmission scan, and the post-drug experiment started about 12:30 p.m. One subject underwent the post-drug experiment on the following day of the control experiment, and another subject underwent the control experiment 3 weeks after the post-drug experiment. Three subjects received a placebo. In the post-drug experiment, the tracer was injected 90 min after the oral administration of the drug. The subjects reported sleepiness about 60 min after the oral administration of triazolam. Blood sampling was performed immediately before the tracer injection to measure the blood level of triazolam.

A memory test was performed at the time of the control and post-drug PET experiments. Subjects were tested with two test batteries, using meaningful combinations of two syllables of "kana" Japanese syllabary, as well as meaningless combinations (Ebbinghaus 1885; Ikeda et al. 1989). Three sets of both tests, with equal difficulty and consisting of five printed cards in each test, were used. Instructions and rehearsals took place before the first experiment. Each card was presented for 4 s followed by the reciting of syllables. Immediate recall of both tests was performed just before the control and post-drug PET experiments, using different sets of tests. At first, five meaningful syllables were presented and subjects were asked to recall them; then five meaningless syllables were presented and subjects were asked to recall. Delayed recall of the meaningful syllables was first carried out immediately after the immediate recall of the meaningless syllables to investigate the interference effect, and again after the PET scan (60 min). Delayed recall of the meaningless syllables was conducted after the PET scan (60 min) following the meaningful syllables. All memory tests were performed in the PET scanner.

During the 60 min following the injection of the tracer, subjects were instructed to keep their eyes open and remain awake. To keep the subjects awake, two different tones were presented via a cassette recorder in a random fashion with one target tone occurring during 40% of the total stimulus presentation. Subjects were instructed to sound a horn when the target tone was heard. If the use of the horn stopped, tactile stimulation was added on the right hand to awaken the subject.

PET. [^{11}C]-labelled *N*-methyl-4-piperidylbenzilate (NMPB) (Mulholland et al. 1988; Koeppe et al. 1992; Suhara et al. 1993), a potent muscarinic cholinergic antagonist for both M_1 and M_2 subtypes (Sokolovsky et al. 1980; Gurwitz et al. 1987), was synthesized by *N*-methylation of 4-piperidylbenzilate with [^{11}C]methyliodide. The radiochemical purities were more than 95%, and the specific radioactivities at the time of injection ranged from 12 to 47 GBq/μmol (Table 1). A whole-body, eight ring positron emission tomography system (Hitachi PCT 3600 W) was used to follow the radioactivity in 15 sections of the brain covering an axial field of view of 100 mm. The intervals between the section midpoints were 7 mm. The spatial resolution for the reconstructed image was 6.5 mm FWHM (full width at half maximum) and the in-plane and cross-plane slice thicknesses were 6.9 mm and 5.9 mm, respectively (Endo et al. 1991).

The head of the subject was carefully aligned parallel to the orbitomeatal (OM) line with the aid of a vertical laser line. The head was immobilized for the duration of the scanning session with a specially designed head holder and a face mask. The face mask was molded to the shape of the face of each subject and fastened to the head holder with plastic bolts to assure accuracy on re-positioning (Suhara et al. 1992).

A transmission scan for attenuation correction was performed using a ^{68}Ge-^{68}Ga source before the first emission scan and this data was also used for the reconstruction of the second emission data. A dose of 333–720 MBq [^{11}C]NMPB was injected intravenously into the antecubital vein with a 10-ml saline flush (Table 1). Thirty sequential scans were performed during a period of 60 min immediately after the injection.

The tissue concentration of carbon 11 in eight brain regions (pons, hippocampus, frontal cortex, striatum, temporal cortex, thalamus, occipital cortex, parietal cortex) was obtained from regions of interest (ROIs) defined on the image of the emission scan. Irregular ROIs were defined by computer-controlled delineation of the percentage isocontour (Suhara et al. 1992) with reference to the brain atlas (Matui and Hirano 1977) and to MRI of young normal volunteers which parallel the OM line of the same slice thickness as the PET image. For each subject, the ROIs were delineated by the same standard and ROIs used in the second experiment were confirmed to be the same as those of the first experiment by comparing images of both ROIs. The average values of left and right ROIs were used for calculation.

Data analysis. The compartment model was applied for the quantification of receptor binding in vivo (Wong et al. 1986). As the cerebellum contains few muscarinic cholinergic receptors (Lin et al. 1986), and a saturation study using a rhesus monkey indicated that 30 μg/kg NMPB, which inhibits more than 80% of the specific binding in the cerebral cortex, did not significantly affect the [^{11}C]NMPB accumulation of radioactivity in the cerebellum (unpublished data), the cerebellum was used as an inert region. Assuming that the rate of the ligand across the blood-brain barrier is the same in the cerebellum as in the rest of the brain and that the ligand is irreversibly

Table 1. Radiochemical data of [^{11}C]NMPB for each experiment and blood concentration of triazolam

Subject	Specific radioactivity (GBq/μmol)		Injected radioactivity (MBq)		Weight of cold ligand (nmol/kg)		Blood concentration of triazolam
	Control	Drug	Control	Drug	Control	Drug	(ng/ml)
Subj. 1	16.1	31.9	333	449	0.38	0.26	3.58
Subj. 2	36.5	15.9	677	438	0.31	0.47	2.6
Subj. 3	27.2	21.8	641	680	0.36	0.48	2.52
Subj. 4	20.5	20.0	580	605	0.47	0.50	3.31
Subj. 5	34.7	12.4	459	497	0.24	0.72	3.17
Subj. 6	28.4	37.6	676	602	0.43	0.29	3.99
Subj. 7	31.0	19.9	720	524	0.38	0.43	placebo
Subj. 8	34.8	21.5	574	638	0.28	0.51	placebo
Subj. 9	46.6	28.4	634	701	0.21	0.28	placebo

bound throughout the duration of the experiment, the ratio between the measured radioactivity in the specific binding region (Ms) and that in the cerebellum (Mc) can be expressed by the following equation (Patlak and Blasberg 1985; Wong et al. 1986; Eckernäs et al. 1987)

$$\frac{Ms(t)}{Mc(t)} = \frac{k_2 k_3}{k_2 + k_3} \frac{\int_0^t Mc(\tau)\, d\tau}{Mc(t)} + \left(\frac{k_2}{k_2 + k_3}\right)^2 + \frac{k_3}{k_2 + k_3} \quad (1)$$

The rate constant k_3 is the association rate constant of the ligand with the receptors, which is equal to the product of the bimolecular association constant (k_{on}) and the number of unoccupied receptors (B'_{max}), whereas k_4 is the rate of dissociation from the receptor and k_2 is the efflux rate constant from tissue to blood (Wong et al. 1986). If $k_2 \gg k_3$, then equation (1) can be written (Wong et al. 1984; Eckernäs et al.1987)

$$\frac{Ms(t)}{Mc(t)} = k_3 \frac{\int_0^t Mc(\tau)\, d\tau}{Mc(t)} + 1 \quad (2)$$

By this method, the $Ms(t)/Mc(t)$ ratio plotted against the normalized integral of the radioactivity in the cerebellum, $\int_0^t Mc(\tau)\, d\tau / Mc(t)$, yields a straight line (Wong et al. 1986; Eckernäs et al. 1987; Suhara et al. 1993). The slope of this line (k_3) was used for the quantification of receptor binding.

Blood concentration of triazolam. A 1-ml plasma sample was diluted with 2 ml 50 mM ammonium acetate buffer (pH 4.0) containing 10 ng alprazolam as an internal standard. The diluted sample was applied to the solid-phase extraction column. The drugs were eluted with 0.5 ml 50% aqueous methanol and were analyzed on an HPLC system.

Results

PET study

Figure 1 shows the time course of [^{11}C]NMPB in both control and post-drug experiments. The accumulation of [^{11}C]NMPB in the frontal cortex was significantly decreased after administration of triazolam. The time course in the cerebellum was not significantly different between the control and the post-drug experiments. Figure 2 shows the decreased slope (k_3) of the frontal cortex after triazolam administration compared with the control experiment. Figures 1 and 2 show the data from subject 1 who underwent the post-drug experiment on the day following the control experiment. The length of interval between control and post-drug experiment or the order of drug administration had no significant effect on the results. The k_3 was decreased after triazolam administration in the pons, hippocampus, frontal cortex, striatum, temporal cortex, thalamus, occipital cortex and parietal cortex (Table 2, Fig. 3). The reduction compared to the control study varied from 8.6±3.7% in the temporal cortex to 16.3±6.3% in the thalamus and all regions

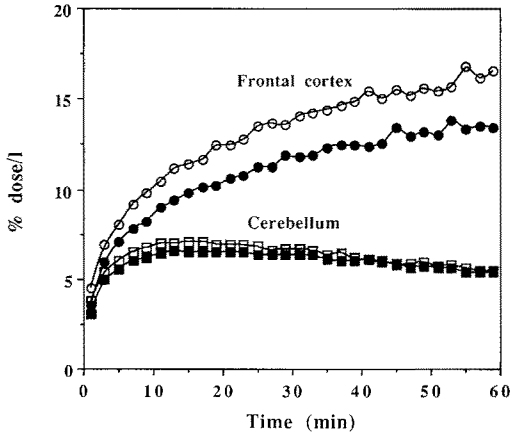

Fig. 1. Time course of [^{11}C]NMPB in the frontal cortex of subject 1. The accumulation of [^{11}C]NMPB in the frontal cortex was significantly decreased after triazolam administration (●) [control (○)]. The time course in the cerebellum was not significantly different between the control (□) and post-drug experiments (■)

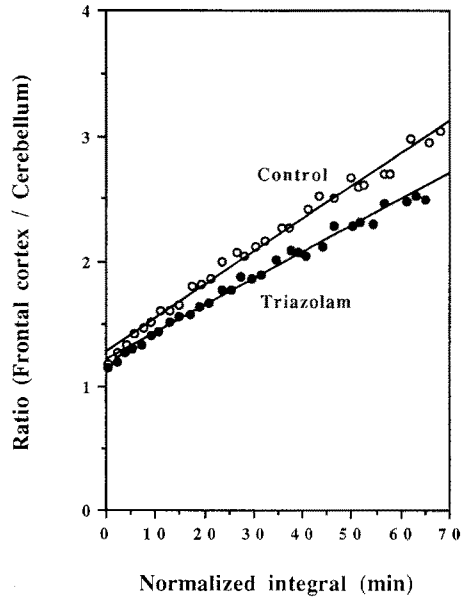

Fig. 2. The ratio of radioactivity in the frontal cortex to that in the cerebellum of subject 1 plotted against the normalized integral of the radioactivity in the cerebellum. The slope (k_3) of the frontal cortex decreased after triazolam administration (●) compared to the control experiment (○)

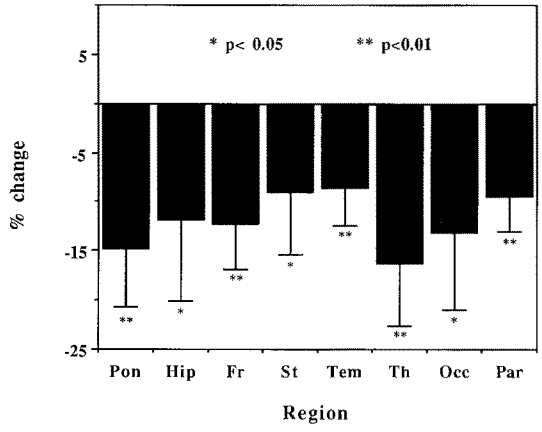

Fig. 3. The percent of k_3 reduction after triazolam administration compared to the control study. The k_3 value was decreased after triazolam administration in the pons (*Pon*), hippocampus (*Hip*), frontal cortex (*Fr*), striatum (*St*), temporal cortex (*Tem*), thalamus (*Th*), occipital cortex (*Occ*) and parietal cortex (*Par*). The percent reduction varied from 8.6±3.7% in the temporal cortex to 16.3±6.3% in the thalamus; all were statistically significant (paired *t* test; $P<0.05$ or $P<0.01$)

Table 2. Effects of triazolam on k_3 (min^{-1}) in eight brain regions

Subject		Pons	Hippo-campus	Frontal cortex	Striatum	Temporal cortex	Thalamus	Occipital cortex	Parietal cortex
Subj. 1	control	0.0170	0.0171	0.0266	0.0259	0.0246	0.0246	0.0276	0.0235
	triazolam	0.0128	0.0162	0.0213	0.0219	0.0220	0.0183	0.0242	0.0204
Subj. 2	control	0.0157	0.0153	0.0225	0.0248	0.0220	0.0176	0.0234	0.0218
	triazolam	0.0140	0.0135	0.0205	0.0236	0.0200	0.0154	0.0213	0.0200
Subj. 3	control	0.0175	0.0171	0.0255	0.0266	0.0245	0.0224	0.0289	0.0237
	triazolam	0.0141	0.0140	0.0217	0.0238	0.0214	0.0180	0.0246	0.0202
Subj. 4	control	0.0167	0.0197	0.0274	0.0290	0.0275	0.0188	0.0253	0.0261
	triazolam	0.0146	0.0176	0.0246	0.0277	0.0244	0.0164	0.0233	0.0244
Subj. 5	control	0.0178	0.0242	0.0310	0.0337	0.0288	0.0203	0.0287	0.0290
	triazolam	0.0157	0.0239	0.0285	0.0331	0.0280	0.0186	0.0268	0.0269
Subj. 6	control	0.0171	0.0207	0.0249	0.0315	0.0241	0.0199	0.0279	0.0222
	triazolam	0.0156	0.0158	0.0220	0.0261	0.0227	0.0161	0.0202	0.0208
Control	mean	0.0170	0.0190	0.0263	0.0286	0.0252	0.0206	0.0270	0.0244
	±SD	±0.0007	±0.0032	±0.0028	±0.0035	±0.0024	±0.0025	±0.0022	±0.0027
Triazolam	mean	0.0145**	0.0169*	0.0231**	0.0260*	0.0231**	0.0171**	0.0234*	0.0221**
	±SD	±0.0011	±0.0037	±0.0030	±0.0040	±0.0028	±0.0013	±0.0024	±0.0029

* $P<0.05$, ** $P<0.01$, Paired t test between control and post-drug k_3 values

Table 3. Effects of placebo on k_3 (min^{-1}) in eight brain regions

Subject		Pons	Hippo-campus	Frontal cortex	Striatum	Temporal cortex	Thalamus	Occipital cortex	Parietal cortex
Subj. 7	control	0.0195	0.0205	0.0288	0.0343	0.0271	0.0218	0.0315	0.0298
	placebo	0.0176	0.0207	0.0278	0.0334	0.0265	0.0207	0.0313	0.0289
Subj. 8	control	0.0185	0.0232	0.0287	0.0295	0.0283	0.0248	0.0315	0.0265
	placebo	0.0171	0.0226	0.0296	0.0308	0.0297	0.0243	0.0329	0.0268
Subj. 9	control	0.0156	0.0239	0.0289	0.0319	0.0280	0.0240	0.0333	0.0270
	placebo	0.0146	0.0225	0.0296	0.0317	0.0271	0.0227	0.0327	0.0264
Control	mean	0.0179	0.0225	0.0288	0.0319	0.0278	0.0236	0.0321	0.0278
	±SD	±0.0020	±0.0018	±0.0001	±0.0024	±0.0006	±0.0016	±0.0010	±0.0018
Placebo	mean	0.0164	0.0219	0.0290	0.0320	0.0278	0.0226	0.0323	0.0274
	±SD	±0.0016	±0.0011	±0.0010	±0.0013	±0.0017	±0.0018	±0.0009	±0.0013

showed statistical significance (paired t test; $P<0.05$ or $P<0.01$) (Fig. 3). There was no significant change in k_3 after placebo administration in eight brain regions (Table 3). The average blood concentration of triazolam immediately before the injection of the tracer was 3.20±0.57 ng/ml. Within this range, no statistically significant relation was observed between the percent reduction of k_3 and blood concentration in any brain regions.

Memory tests

Table 4 shows the average correct responses for immediate and delayed recall. Triazolam administration impaired both immediate and delayed recall of meaningful and meaningless syllables (paired t test; $P<0.05$, $P<0.01$ or $P<0.001$), whereas placebo administration did not have an effect on either immediate or delayed recall. However, the deficits were more pronounced in delayed

Table 4. Effects of triazolam and placebo on immediate and delayed recall of meaningful and meaningless syllables

	Triazolam		Placebo	
	Control	Triazolam	Control	Plecabo
Immediate recall				
Meaningful	5.0	3.5±1.4*	5.0	5.0
Meaningless	4.2±0.8	1.8±1.2*	3.7±0.6	4.0±1.0
Delayed recall				
Meaningful (pre-scan)	4.5±0.5	0.7±0.8***	5.0	4.6±0.6
Meaningful (post-scan)	4.1±0.8	0.2±0.4***	5.0	4.0±1.0
Meaningless	2.7±1.0	0**	2.0±1.0	2.3±1.2

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ paired t test between control and post-drug results
Values are mean±SD

recall than in immediate recall. The first delayed recalls (before PET scan) were severely affected by the interference of meaningless syllables. Subjects who performed two tests on the same day recalled the syllables presented in the control experiments (2.5 ± 1.9), rather than the syllables presented after triazolam administration at the post-drug delayed recall. There was no statistically significant correlation between the percent of reduction of k_3 and the correct number of recall responses in the analyzed brain regions.

Discussion

In this study we demonstrated that acute treatment with triazolam decreased the specific binding of [^{11}C]NMPB in vivo in the human brain and impaired memory. The post-triazolam decrease in the binding is probably drug induced, as the placebo group did not show a similar change.

Benzodiazepine treatment is reported to reduce the cerebral blood flow, predominantly in the subcortical regions such as the striatum and cerebellum (Matthew et al. 1991). The degree of reduction ranges from 5 to 30% depending on the type of drugs used in the experiment and the methods (Mathew et al. 1985; Matthew et al. 1991). In the present study, a decrease in k_3 of [^{11}C]NMPB due to changes in cerebral blood flow following administration of triazolam cannot be ruled out, when the magnitude of k_3 is significant compared to the magnitude of k_2 (Patlak and Blasberg 1985). However, it is unlikely that the decrease in k_3 was simply due to changes in the cerebral blood flow, because the time course of the cerebellum, which contains the flow component used as a reference region in this study, was not significantly changed by triazolam administration, especially after 30 min when steady state levels between plasma and cerebellum were expected.

A metabolite study using a thin-layer chromatographic method indicated that in mice more than 95% of the radioactivity in the brain was unmetabolized [^{3}H]NMPB at 60 min after the injection (unpublished data). Generally, the rate of drug metabolism is much slower in humans than in mice, and it is thus unlikely that the metabolites of [^{11}C]NMPB affected the present results.

As benzodiazepines do not bind to muscarinic receptors directly (Nilvebrant and Sparf 1986), this change in muscarinic receptor binding is explained by the indirect effect of triazolam in the in vivo condition. Numerous experiments have demonstrated that the activity of the forebrain cholinergic neurons is modulated by inhibitory GABA-benzodiazepine system (Sarter et al. 1990). Benzodiazepine agonists are reported to decrease the cortical acetylcholine release (Consolo et al. 1974; Petkov et al. 1983). The decrease of acetylcholine release in the synaptic cleft may explain the decreased binding of [^{11}C]NMPB, since endogenous neurotransmitters such as dopamine modulate the in vivo binding of the dopamine antagonist [^{3}H]spiperone, which is irreversibly bound to the receptors during the study, in the same direction as the dopaminergic activity (Chugani et al. 1988; Inoue et al. 1991a). In other words, the higher the dopamine in the synaptic cleft, the higher the binding of [^{3}H]spiperone to the receptor; likewise, the lower the dopamine concentration, the lower the binding (Bischoff et al. 1991). Agonist-mediated receptor internalization is reported to be the explanation of this phenomenon, which is the opposite of the effect expected from in vitro studies (Chugani et al. 1988). However, opposite results are reported using the reversible binding ligand, raclopride (Ross and Jackson 1989; Farde et al. 1992; Hume et al. 1992) or even the irreversible binding ligand *N*-methylspiperone (Dewey et al. 1991; Young et al. 1991). These discrepancies are partly explicable by the variations in individual responses to the drugs or by the difference in experimental conditions. However, the difference of the kinetic properties of the ligands seems more important, since the effect of reserpine on receptor binding in vivo acts in an opposite manner to the reversible binding ligand [^{3}H]raclopride and the irreversible binding ligand [^{3}H]*N*-methylspiperone (Inoue et al. 1991b). NMPB has a much higher affinity to muscarinic cholinergic receptors than acetylcholine itself ($K_D = 0.41$ nM, $IC_{50} = 30$ μM for acetylcholine) (Sokolovsky et al. 1980) and here it irreversibly bound to the receptors in the brain during the study. If the role of endogenous acetylcholine on [^{11}C]NMPB binding is the same as in irreversible dopaminergic ligand binding, the decrease in [^{11}C]NMPB binding might be explained by the decrease of acetylcholine release. However, there are many unknown mechanisms in ligand-receptor binding in vivo; there is a possibility that other factors which modulate the rate-limiting step of ligand receptor binding in vivo might be changed by triazolam administration (Inoue et al. 1992).

In confirmation of previous studies, the present results of the memory test show that significant anterograde memory deficit occurs following triazolam administration (Roth et al. 1980). Although triazolam did affect the immediate recall, the delayed recall was more greatly affected. The present results show that triazolam-treated subjects could recall the syllables presented before triazolam administration at the post-drug delayed recall, even though the subject could not recall syllables presented after triazolam administration. The amnesia caused by benzodiazepine is reported to be a failure of acquisition and consolidation of new information rather than retention and retrieval (Lister 1985; Dorow et al. 1987). The present results are consistent with this view, and also indicate a significant deficit after the interference task of the meaningless syllables. The interference effect is reported to be related to a disorder of the medial temporal lobe (Hermann et al. 1988). Furthermore, the medial temporal lobe, thalamus and frontal basal region have been reported to be associated with memory (Fazio et al. 1992). However, even though there are some regional differences in the reduction of [^{11}C]NMPB binding, the present results indicate a rather diffuse effect of triazolam in the brain. These results might be explained by the diffuse interaction between triazolam and the cholinergic system; that is, not only memory and cognitive function, but also sedation, sleep and motor function.

In this study, we have demonstrated the benzodi-

azepine/cholinergic interactions in the human brain using PET and confirmed the amnestic effect of triazolam. However, further studies are needed to clarify the mechanism of in vivo receptor binding, and more elaborate clinical evaluations are needed to assess the relationship between the PET data and clinical symptoms. Even though there are many unknown crucial factors in the in vivo receptor binding, to measure the interaction between the different neural systems using in vivo receptor binding is important to elucidate the mechanism of drug action in the central nervous system (Dewey et al. 1990; Suhara et al. 1990).

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